Aus dem Institut für Tierzucht und Tierhaltung der
Agrar- und Ernährungswissenschaftlichen Fakultät der
Christian-Albrechts-Universität zu Kiel

NEW APPROACHES TO ENHANCE THE ENDOGENOUS LC-PUFA BIOSYNTHESIS IN RAINBOW TROUT

Dissertation

zur Erlangung des Doktorgrades

der Agrar- und Ernährungswissenschaftlichen Fakultät

der Christian-Albrechts-Universität zu Kiel

vorgelegt von

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(Master of Science Agrarwissenschaften)

geboren in Memmingen, Deutschland

Kiel, 2018

Dekan:	Prof. Dr. Dr. Christian Henning
Erster Berichterstatter:	Prof. Dr. Carsten Schulz
Zweiter Berichterstatter:	Prof. Dr. Gerald Rimbach
Tag der mündlichen Prüfung:	23. Januar 2019

Die vorliegende Arbeit wurde mit dankenswerter finanzieller Unterstützung aus dem Bundesprogramm Ökologischer Landbau und andere Formen nachhaltiger Landwirtschaft (BÖLN) der Bundesanstalt für Landwirtschaft und Ernährung (BLE) angefertigt.

Gedruckt mit Genehmigung der Agrar- und Ernährungswissenschaftlichen Fakultät der Christian-Albrechts-Universität zu Kiel.

Für meine Eltern

Julianna und Josef

Sage ja zu den Überraschungen, die deine Pläne durchkreuzen, deine Träume zunichtemachen, deinem Tag eine andere Richtung geben – ja vielleicht deinem Leben. Sie sind kein Zufall.

(Helder Camara)

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GENERAL INTRODUCTION

The growth of the global human population and a change in human dietary preferences towards more animal products, especially in Asian countries, has increased the demand for aquaculture products globally [1]. In addition, the limited supply of fish for human consumption from world capture fisheries contributed indirectly to the increased aquaculture production. It is estimated, that the production from world capture fisheries will stagnate on a level of around 90 million metric tons per year [2]. In addition, only approximately 10.5% of marine fish stocks are currently considered to be underfished [2]. Thus, as global human population growth will continue, any further fish available for human consumption will have to come mainly from aquaculture production. A report from the World Bank estimates that in the year 2030 aquaculture production will provide over 60% of the fish for human consumption [3].

Intensively farmed fish species rely on compound aquafeeds with optimal physical and chemical qualities which are adapted to the fish to achieve a high product quality for human nutrition. Due to the increased demand for fish, compound aquafeed production increased rapidly from 7.6 million tons in 1995 [4] to 49.6 million tons in 2016 and is predicted to expand further by reaching 76.2 million tons in 2025 [5]. In the European Union, mainly carnivorous fish species like Atlantic salmon, rainbow trout and gilthead seabream are cultured [6]. These fish species require energy-dense feeds [7]. Most carnivorous fish species only have a poor capability to digest carbohydrates (reviewed in [8]). Thus, dietary lipids are utilized as the major energy contributors to avoid the oxidation of valuable and expensive protein for energy supply [9,10]. Traditionally, fish oil was used as the main lipid source in diets for fish species like Atlantic salmon and rainbow trout [11,12]. Besides being a source of energy, fish oil is rich in omega-3 (n-3) long chain polyunsaturated fatty acids (LC-PUFA) like eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) [13]. In the past, the utilization of fish oil was economically reasonable, as it was inexpensive and readily available [11,14]. However, the current state is different: The worldwide production of fish oil peaked in 1987 and 1990 with 1.6 million metric tons per year [7]. Since 2005, it declined steadily, and was less than 1 million metric tons in 2015 [15]. In addition, climate change impacts might promote a more frequent occurrence of El Niño [16]. This weather phenomenon has already caused a decrease in the population of small pelagic fish such as anchovies [17,18], the primary source for fish oil production [12,19]. In addition, already 75% of the fish oil produced worldwide is used for aquafeeds mainly for salmonids and marine finfish species (reviewed in [19]). The limited availability concomitant with the high demand excessively increased its price [11]. Aquafeeds for farmed fish make up approximately 50-80% of the total production costs in intensive aquaculture systems [7]. Depending on fish oil as a feed ingredient, its price increase became a bottleneck for the economic and sustainable growth of the industry [21].

Vegetable oils and fatty acids in fish nutrition

A lot of research has been conducted so far to replace fish oil with vegetable oils in salmonid diets (reviewed in [22]). Vegetable oils are available on a large scale, cost effective and contain significant amounts of α-linolenic acid (18:3n-3, ALA) and linoleic acid (18:2n-6, LA) [13,23,24]. The content of these C18 fatty acids is important, since fish, like all vertebrates, lack the delta-12 and delta-15 desaturases that are needed to synthesize ALA and LA de novo from 18:1n-9 [25]. Thus, fish rely on the dietary uptake of these fatty acids. Further physiologically essential fatty acids are the longer chained fatty acids arachidonic acid (20:4n-6, ARA), EPA and DHA [25,26]. Whether fish depend on the dietary supply or whether they are able to endogenously synthesize ARA, EPA and DHA from their PUFA precursors is species specific [27]. It depends on the presence and expression of various enzymes involved in the LC-PUFA biosynthesis like the delta-5- and delta-6-desaturase as well as the elongases [25,26]. In contrast to many marine fish species, many freshwater species are able to synthesize EPA and DHA from ALA. For example, salmonids like rainbow trout rely on their capability of endogenously synthesizing EPA and DHA as their natural prey is not rich in these fatty acids but contains high amounts of ALA and LA [25]. Thus, salmonids can be fed on plant-based diets without growth impairment as long as the requirement levels of ALA and LA are met [28–31]. This fact has made vegetable oils the most implemented alternative to fish oil in salmonid diets with inclusion levels from 0% in 1990 to 19.2% in 2013 [12]. However, the fatty acid biosynthesis is not able to compensate for the lack of dietary EPA and DHA in plant-based diets, leading to significantly lower LC-PUFA tissue levels in comparison to fish fed dietary fish oil [28,30,32]. Thus, although dietary vegetable oils are able to satisfy the nutritional requirements for rainbow trout and can be considered economically and ecologically sustainable, the product quality of these fish is decreased for human consumption [33].

Fish in human nutrition

Fish plays an important role in human nutrition. It provides about 17% of the worldwide available animal protein for human consumption [34]. In addition, fish is exceptionally rich in vitamin D [35] which is important for bone health [36]. Further, fish is a unique natural source of iodine and contains considerable amounts of selenium [35]. However, the main health-beneficial effects are associated with the LC-PUFA content, specifically the content of EPA and DHA. EPA and DHA have been shown to be important for the infant neuronal and retinal development as well as the immune function [37,38]. Further, these fatty acids play a positive role in cardiovascular diseases, cancer and various mental illnesses (e.g. dementia) [38,39]. Similar to some fish species, humans are able to biosynthesize EPA and DHA from ALA. However, only 8% of ALA is converted to EPA and only 0.05-4% to DHA (reviewed in [40]). Moreover, high levels of n-6 fatty acids can additionally reduce the conversion efficiency by

40-50% (reviewed in [41]). The optimal n-6:n-3 ratio in human nutrition is approximately 1:1. However, in developed countries the dietary intake of n-6 fatty acids as well as the content of total fat and saturated fatty acids has increased, leading to a shift of the n-6:n-3 ratio toward 20:1 which increases the risk of coronary heart disease and diabetes among other ailments (reviewed in [42]). Thus, to counteract the negative effects of an increased n-6:n-3 ratio on human health, the daily intake of up to 650 mg EPA and DHA via two servings of fish per week is recommended (reviewed in [43]). The LC-PUFA content in fish is dependent inter alia on the factors species, lipid content of flesh and dietary fatty acid composition [33,44–46]. Fish like Atlantic salmon and rainbow trout can be considered as rather oily and rich in EPA and DHA [33,47,48]. The main source of LC-PUFA in these fish is based on the supply via dietary fish oil and fishmeal [20,49]. The substitution of marine feed ingredients by terrestrial ingredients has caused a major effect in the product quality of fish for human nutrition. A study of farmed Atlantic salmon has shown a decrease of EPA and DHA levels from 2005 to 2015, which is due to the inclusion of dietary vegetable oils [33]. Thus, there is a major shortfall in EPA and DHA and the supply of these fatty acids by fish is far lower than the demand for the requirement for the global population.

Strategies to ensure high quality fish for human consumption

Significant effort has been put into finding alternatives to fish oil. For example, microalgae are the primary producers of LC-PUFA in the marine environment [23] and are already used as dietary supplements in human nutrition [34]. However, the production of fish oil alternatives has mainly been limited to DHA-producing, heterotrophic species (e.g. *Schizochytrium*) [50]. Their production is, so far, only possible in low volumes with high costs [23]. However, the genes of specific microalgae encoding for enzymes involved in the biosynthesis of EPA and DHA can be used in genetically modified (GM) oil crops. These genes have been inserted into plants such as camelina (*Camelina sativa*) and soy (*Glycine max*) generating seeds with up to 20% of n-3 LC-PUFA [51,52]. For example, genetically modified camelina oil containing about 6% EPA and DHA each has been shown to be a potent candidate to substitute dietary fish oil in Atlantic salmon. In this study, fish fed the diet containing camelina oil showed no difference in growth and health parameters compared to salmon fed other dietary treatments. Further, n-3 LC-PUFA tissue levels were higher compared to fish fed a diet based on an oil from a wild-type of camelina [51]. However, genetically modified oilseed crops are currently not produced on a commercial scale and their utilization as feed ingredients in aquafeeds is still controversial [53,54]. Other strategies, like selective breeding for the heritable trait of n-3 LC-PUFA level in Atlantic salmon has been evaluated [55]. In addition, new feeding strategies like nutritional programming and circadian feeding schedules to promote the EPA and DHA contents were investigated [56,57]. For example, gilthead sea bream were fed diets based on canola oil and fish oil in daily alternation. These fish showed enhanced growth, feed intake and higher EPA

and DHA tissue levels compared to fish fed either of these diets continuously [57]. Another, commercially applied, feeding strategy is the use of finisher diets [58]. A fish oil based-diet is fed during the grow-out phase in order to restore the fatty acid contents in fish fed vegetable oil-based diets during the ongrowing phase to the desired n-3 LC-PUFA concentrations for human nutrition [30,58]. Although, the total amount of fish oil is reduced by this strategy, there is still a dependence on fish oil as a feed ingredient.

This thesis will focus on the utilization of bioactive substances and a stearidonic acid-rich oil, respectively to substitute fish oil.

Bioactive substances

Isoflavones belong to the group of bioactive substances and naturally occur as glycosides in tropical legumes like soy and red clover (*Trifolium pratense*) [59,60]. In the intestine, isoflavone glycosides are hydrolyzed by various enzymes and are either absorbed or further metabolized. Daidzein, for example, can be metabolized to equol by the intestinal bacterial metabolism in animals and humans (reviewed in [61]). In general, bioactive substances have health-beneficial effects beyond their basic nutritional value in human nutrition. For example, isoflavones have been associated with a reduced occurrence of breast and prostate cancer in humans of Asian countries due to their plant-based diet (reviewed in [62]). Besides their potential health benefits in humans, they might also be able to stimulate the endogenous biosynthesis of EPA and DHA and thereby increase the tissue levels of these fatty acids in rainbow trout. Genistein and daidzein have been shown to improve the lipid metabolism in obese-zucker rats and increase the expression of the transcription factor peroxisome proliferator-activated receptor alpha (PPARα) in murine cells [63]. PPARα is centrally involved in the peroxisomal β-oxidation [64] necessary to obtain DHA [20]. Further, soy isoflavones affected the lipid metabolism in rats (reviewed in [65]) and fish [66]. For example, genistein significantly increased the expression of genes involved in the biosynthesis pathway of n-3 LC-PUFA in hepatocytes of Atlantic salmon [66]. Due to their structural similarity to estrogen, isoflavones belong to the group of phytoestrogens [60]. Thus, these substances and the metabolite equol are able to bind to estrogen-receptors and mimic estrogen [67–70]. Genes encoding proteins involved in the n-3 LC-PUFA synthesis pathway can be affected by estrogen [71–74]. This could be an important factor to increase tissue n-3 LC-PUFA levels in rainbow trout.

Stearidonic acid-rich oils

In contrast to common plants, *Buglossoides arvensis*, *Echium plantagineum* and *Ribes nigrum* naturally contain stearidonic acid (18:4n-3, SDA). The levels of SDA in the oils of these plants depend on the species and are in the range of 3% (*Ribes nigrum*) [75] to ~13% (*Echium*

plantagineum) and up to 18% (*Buglossoides arvensis*) [22]. These plants have a gene encoding for the delta-6-desaturase [23]. Thus, they are able to synthesize SDA from ALA and 18:3n-6 from LA, respectively. In vertebrates, this step has been considered to be rate limiting in the LC-PUFA synthesis (reviewed in [45]). Thus, bypassing the initial synthesis step can increase the overall efficiency of the synthesis. This has already been shown in humans [76,77] and animals [78,79] leading to increased EPA levels. However, dietary Echium oil lead to controversial results in salmonids [80–82]. For example, rainbow trout fed a diet based on Echium oil showed similar LC-PUFA tissue levels as fish fed a diet based on linseed oil, as the substrate (ALA/SDA) availability might also be a limiting factor of the biosynthesis [80]. Ahiflower oil, an oil of the plant *Buglossoides arvensis*, is rich in SDA but has also significant amounts of ALA [83]. Thus, dietary Ahiflower oil might increase EPA and DHA tissue levels in rainbow trout by providing both SDA, to increase the efficiency of the biosynthesis, and ALA for further substrate availability.

Aim of the thesis

As mentioned in the beginning, a compound aquafeed has to ensure optimal physical and chemical quality adapted to the fish to achieve a high product quality for human nutrition. Although, these requirements might not have changed substantially over the last decades, it is more difficult than ever to meet them nowadays. Due to the limited availability of fish oil, the aquaculture production faces massive challenges in finding adequate alternatives to this feed ingredient. The approaches in this thesis aimed to enhance the levels of EPA and DHA in rainbow trout which were fed vegetable oil-based diets in order to increase the nutritional value of these fish for human consumption. The approaches are built on three hypotheses:

(1) Isoflavones and their metabolites are able to stimulate the endogenous biosynthesis of LC-PUFA
(2) Dietary *Buglossoides arvensis* (Ahiflower) oil enhances the efficiency of the LC-PUFA biosynthesis
(3) Combining isoflavones and Ahiflower oil leads to an interaction that further increases the levels of LC-PUFA

In total, four experiments were conducted to test these hypotheses. All experiments were performed with rainbow trout as this fish species is known for their ability to synthesize EPA and DHA endogenously.

Chapter 1 examines the potential of the bioactive substances bioachanin A, daidzein, genistein and equol to stimulate the biosynthesis of EPA and DHA in rainbow trout and discusses the following research questions:

- Are they able to stimulate the biosynthesis of EPA and DHA and thereby increase the content of these fatty acids in tissues of rainbow trout fed with a vegetable oil-based diet?
- Do these substances affect growth of fish and nutrient composition of whole body homogenates of rainbow trout?
- Do they alter the expression and protein levels of genes encoding proteins involved in the fatty acid biosynthesis?

Chapter 2 focuses on the dose-response of the most promising substances of **chapter 1** (genistein and equol) in order to increase the efficiency of the biosynthesis of EPA and DHA in rainbow trout and addresses the following research aims:

- Do different dietary concentrations of genistein and equol affect growth of fish and nutrient composition of whole body homogenates of rainbow trout differently?
- Are higher/lower dietary dosages of these substances more efficient in increasing tissue levels of EPA and DHA?
- Do these substances and the dietary dosages, respectively, affect the fatty acid composition of tissues (liver, whole body homogenate and fillet) differently?

Chapter 3 discusses the suitability of dietary Ahiflower oil to enhance EPA and DHA in rainbow trout tissues and examines the following scientific issues:

- Do increased dietary ALA and SDA levels increase the efficiency of the EPA and DHA biosynthesis in rainbow trout?
- Which concentration of Ahiflower oil can be used in rainbow trout diets without impairing growth performance and nutrient composition of fish?
- Does the inclusion of Ahiflower oil modify the expression of genes involved in the biosynthesis of fatty acids differently in response to the dietary dosage?

Chapter 4 is based on the observations of the **chapters 1-3** and aims to evaluate the interactions of both dietary equol and Ahiflower oil to improve the nutritional quality of rainbow trout, focusing on the following questions:

- Is the combination of dietary Ahiflower oil and different levels of equol able to further increase EPA and DHA levels in rainbow trout?
- Do these dietary treatments have an effect on growth performance of fish?
- Does the combination of dietary Ahiflower oil and equol alter the nutrient composition of whole body homogenates and fillets?
- Which role does the concentration of equol combined with Ahiflower oil play on the hepatic mRNA steady state levels of the delta-6-desaturase?

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CHAPTER 1

Screening dietary biochanin A, daidzein, equol and genistein for their potential to increase DHA biosynthesis in rainbow trout (*Oncorhynchus mykiss*)

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Modified version published in *PLOS ONE*, 2019, 14 (1): 1-18, https://doi.org/10.1371/journal.pone.0210197.

Abstract

Plant oil utilization in aquafeeds is still the most practical option, although it decreases the content of the nutritionally highly valuable omega-3 fatty acids eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) in fish. Phytoestrogens and their metabolites are putatively able to affect genes encoding proteins centrally involved in the biosynthesis of EPA and DHA due to their estrogenic potential. Thus, the aim of the study was to screen the potential of the phytoestrogens to stimulate the biosynthesis of EPA and DHA in rainbow trout (*Oncorhynchus mykiss*). Additionally, the potential effects on growth performance, nutrient composition and hepatic lipid metabolism in rainbow trout were investigated. For that, a vegetable oil based diet served as a control diet (C) and was supplemented with 15 g/kg dry matter of biochanin A (BA), daidzein (DA), genistein (G) and equol (EQ), respectively. These five diets were fed to rainbow trout (initial body weight 83.3 ± 0.4 g) for 52 days. Growth performance and nutrient composition of whole body homogenates were not affected by the dietary treatments. Furthermore, feeding EQ to rainbow trout significantly increased DHA levels by +8 % in whole body homogenates compared to samples of fish fed the diet C. A tendency towards increased DHA levels in whole body homogenates was found for fish fed the diet G. Fish fed diets BA and DA lacked these effects. Moreover, EQ and G fed fish showed significantly decreased hepatic mRNA steady state levels for fatty acyl desaturase 2a (delta-6) (*fads2a(d6)*). In contrast, carnitine palmitoyl transferases 1 (*cpt1*) hepatic mRNA steady state levels and hepatic Fads2a(d6) protein contents were not affected by the dietary treatment. In conclusion, when combined with dietary vegetable oils, equol and genistein seem to stimulate the biosynthesis of DHA and thereby increase tissue DHA levels in rainbow trout, however, only to a moderate extent.

Keywords: Fish, bioactive, phytoestrogen, DHA, biosynthesis, vegetable oil

Introduction

The amount of fish oil in feed formulas decreased steadily over the last decades due to increasing prices, resulting in the increased inclusion of plant oils [1,2]. Despite this fact, the share of aquaculture on global fish oil utilization increased, with most of it used for salmonid compound feeds [3]. On the one hand, this is due to the expanded production of these species in aquaculture [4]. On the other hand, producing alternatives high in eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), such as single cell oils, is still not feasible on a commercial scale due to unreasonably high costs [5,6]. Furthermore, the industry is driven by consumer expectations of farmed fish being a rich source of EPA and DHA [3,5].

In contrast to many other species, salmonids could be reared successfully on diets with up to 100% fish oil replacement [7–11]. Particularly, rainbow trout (*Oncorhynchus mykiss*) can cope with plant oil based diets due to their ability to endogenously biosynthesize omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA) [12]. Thereby, dietary α-linolenic acid (18:3n-3, ALA), which is found in plant oils like linseed and rapeseed oil [13], is converted to the fatty acids EPA and DHA [14]. This, however, is insufficient to maintain the n-3 LC-PUFA levels of fish fed with fish oil [7,15,16]. Nonetheless, the use of plant oils as alternatives is the most practical and implemented option [17].

Dietary application of phytochemicals seems to be a promising option to counteract the negative effect of plant oil inclusion on the n-3 LC-PUFA content in rainbow trout. Phytochemicals include isoflavones, for example biochanin A, genistein and daidzein [18]. Biochanin A is one of the major isoflavones in red clover (*Trifolium pratense*) [19] whereas genistein and daidzein are naturally present in soy (*Glycine max*) [18]. In contrast, equol is not plant-derived but is a metabolite from daidzein [20]. Equol can be synthesized from daidzein by the intestinal bacterial metabolism in animals and humans [21,22]. Isoflavones and their metabolites can act as antioxidants in humans [23] and showed anti-inflammatory properties in human endothelial cells [24].

Biochanin A, genistein, daidzein and equol belong to the group of phytoestrogens and their metabolites, respectively [20]. The estrogenic potency seems to be attributed to their structural similarity to estradiol [25]. This is of particular interest, since genes encoding proteins involved in the n-3 LC-PUFA biosynthesis were found to be responsive to estrogen [26–28]. For example, hepatic delta-6-desaturase expression was upregulated in response to estradiol in rats [26,29]. The delta-6-desaturase is necessary inter alia for synthesizing DHA out of EPA [30]. Kitson et al. [26] suggest that the upregulation of the hepatic delta-6-desaturase expression increased DHA levels in rats. In addition, isoflavones can be potent ligands for the peroxisome proliferator-activated receptor α (PPARα) [31,32]. Carnitine palmitoyl transferase

(CPT) 1 is one of the target genes of PPARα and is centrally involved in the biosynthesis of fatty acids [33].

The objective of this study was to screen the potential of dietary isoflavones (Biochanin A, daidzein, genistein) and their metabolites (equol) to stimulate n-3 LC-PUFA biosynthesis and the potential effects on growth in rainbow trout. For this purpose, a feeding trial was conducted with rainbow trout. A vegetable oil based diet was supplemented with the different isoflavones and equol, respectively. Influences of the different dietary treatments on growth performance, fatty acid composition of liver, whole body and fillet, nutrient composition of whole body as well as hepatic fatty acyl desaturase 2a (delta-6) (*fads2a(d6)*) and *cpt1* mRNA steady state levels and hepatic Fads2a(d6) protein levels were investigated.

Materials and Methods

Experimental setup

The feeding trial was performed at the facilities of the Gesellschaft für Marine Aquakultur mbH (GMA, Büsum, Germany). A total of 300 monosex female rainbow trout juveniles (Forellenzucht Trostadt GbR, Reurieth, Germany) were acclimated to the experimental conditions in a recirculation aquaculture system (RAS, 20 m^3, turnover rate 2.4 h^{-1}, technical oxygen supply). The water purification system consisted of drum filter, biofilter, ultraviolet disinfection and protein skimmer with ozone. A light/dark (12 h/12 h) cycle was adapted. Following a 3-week adaptation period feeding a commercial diet, fish (83.3 ± 0.4 g) were randomly and equally stocked among 15 tanks (volume 150 L, 20 fish per tank, total initial biomass 1666.4 ± 7.2 g). Dietary treatments were randomly distributed in triplicate and were hand fed once per day for 52 days. To adjust daily feed supply of 2.0% of biomass, all tanks were bulk weighed every 14 days. Temperature (16.0 ± 0.6°C), oxygen (10.4 ± 0.2 mg/L O_2) and pH (7.4) were monitored continuously. NH_4^+ (0.5 ± 0.4 mg/L), NO_2^- (0.5 ± 0.2 mg/L) (Microquant test kit for NH_4^+ and NO_2; Merck KGaA, Darmstadt, Germany) and salinity (6.2 ± 0.2 ‰) were measured daily. The experiment was conducted according to the national regulations for animal welfare (TierSchVersV) and the EU Directive 2010/63/EU for animal experiments. Furthermore, it was approved by the Ministry of Energy, Agriculture, the Environment, Nature and Digitalization (MELUND, Kiel, Germany; approved on 11 July 2017, project number V241-37421/2017).

Experimental diets

All diets were formulated based on the same feed ingredient composition, only differing in the supplementation of the bioactive substances. A blend of palm fat, rapeseed, linseed and sunflower oil was used as dietary oil source (Table 1-1).

Table 1-1. Ingredients and nutrient composition (in g/kg dry matter (DM)) of the experimental diets C, BA, DA, EQ and G.

Diet	C	BA	DA	EQ	G
Ingredients [g/kg DM]					
Fish meal [a]	150	150	150	150	150
Blood meal [b]	50	50	50	50	50
Feather meal [c]	55	55	55	55	55
Pea protein isolate [d]	150	150	150	150	150
Soy protein concentrate [e]	50	50	50	50	50
Wheat gluten [f]	140	140	140	140	140
Gelatin [g]	15	15	15	15	15
Wheat starch [f]	210	210	210	210	210
Oil sources	**115**	**115**	**115**	**115**	**115**
Rapeseed oil [h]	36	36	36	36	36
Linseed oil [i]	34	34	34	34	34
Palm fat [j]	29	29	29	29	29
Sunflower oil [h]	16	16	16	16	16
Vitamin Mineral premix [k]	10	10	10	10	10
Calcium hydrogen phosphate [l]	5.0	5.0	5.0	5.0	5.0
α-Cellulose [m]	15	15	15	15	15
Lysine [n]	4.0	4.0	4.0	4.0	4.0
Methionine [o]	1.0	1.0	1.0	1.0	1.0
Bentonite [p]	30	28.5	28.5	28.5	28.5
Biochanin A [q]	-	1.5	-	-	-
Daidzein [q]	-	-	1.5	-	-
Equol [q]	-	-	-	1.5	-
Genistein [q]	-	-	-	-	1.5
Nutrient composition [% DM]					
Dry matter [in % of diet]	89.1	88.2	88.8	89.3	89.0
Crude protein	53.2	53.5	52.7	52.6	53.5
Crude lipid	16.6	16.8	16.6	16.6	16.7
Crude ash	6.0	5.8	5.8	5.8	5.9
Total COH [in % DM] [r]	24.2	23.9	24.9	25.0	24.0
Gross energy [MJ/kg DM]	23.4	23.4	23.3	23.3	23.3

[a] Lean fish meal "low ash", Bioceval GmbH & Co. KG, Cuxhaven, Germany; [b] Daka porcine bloodmeal, Daka Denmark A/S, Løsning, Denmark; [c] GePro Goldmehl, GePro Geflügel-Protein Vertriebsgesellschaft mbH & Co. KG, Diepholz, Germany; [d] Emsland-Stärke GmbH, Emlichheim, Germany; [e] Euroduna Food Ingredients GmbH, Barmstedt, Germany; [f] KRÖNER STÄRKE GmbH, Ibbenbüren, Germany; [g] Gustav Ehlert GmbH & Co. KG, Verl, Germany; [h] Food store, Büsum, Germany; [i] Makana Produktion und Vertrieb GmbH, Offenbach a.d. Queich, Germany; [j] DF 100 PT-PV; EFG Elbe Fetthandel GmbH, Geesthacht, Germany; [k] Emsland-Aller Aqua GmbH, Golßen, Germany; [l] JRS Pharma GmbH & Co. KG, Rosenberg, Germany; [m] Mikro-Technik GmbH & Co. KG, Bürgstadt am Main, Germany; [n] Biolys, Evonik Industries AG, Essen, Germany; [o] MetAmino, Evonik Industries AG, Essen, Germany; [p] Castiglioni Pes y Cía, Buenos Aires, Argentina; [q] Biochanin A: Cas No: 491-80-5, Daidzein: Cas No: 486-66-8, Equol: Cas No: 531-95-3, Genistein: Cas No: 446-72-0, Xi'an Natural Field Bio-Technique Co., LTD, Xi'an Shaanxi, China; [r] Total COH = 100 – (crude protein + crude lipid + crude ash).

These oil sources were included into the diets to meet the dietary ALA requirement (0.7-1.0% of dry diet) of rainbow trout [13]. Furthermore, only 15% of lean fish meal was used to obtain dietary EPA and DHA levels below the recommended levels for these fatty acids (0.4-0.5% of dry diet) for rainbow trout [13]. Each of the dietary treatments was supplemented with either 1.5 g/kg of dry matter biochanin A (BA), daidzein (DA), equol (EQ) or genistein (G), respectively, in exchange for bentonite. A diet without the addition of bioactive compounds was designed as control diet (C), resulting in five experimental diets in total (C, BA, DA, EQ and G). All diets were isoenergetic and isonitrogenous. Dietary fatty acid composition is shown in Table 1-2. Amino acid content (not shown) of the diets was calculated based on the amino acid contents of the single ingredients. The experimental diets were designed following the amino acid requirements of rainbow trout [13]. Diets were produced with a pelleting machine (Type 14U175, Amandus Kahl, Hamburg) with 6 mm length and 4 mm in diameter.

Table 1-2. Fatty acid composition (in % of fatty acid methyl ester (FAME)) of the experimental diets C, BA, DA, EQ and G.

[% of FAME]	C	BA	DA	EQ	G
14:0	0.7	0.7	0.7	0.7	0.7
16:0	21.3	21.2	21.3	21.2	21.3
18:0	3.6	3.6	3.6	3.6	3.6
Total SFA [1]	**26.6**	**26.4**	**26.5**	**26.4**	**26.5**
16:1	0.5	0.6	0.5	0.6	0.6
18:1	28.3	28.4	28.4	28.4	28.3
Total MUFA [2]	**32.6**	**32.6**	**32.6**	**32.7**	**32.6**
n-6					
18:2n-6	22.4	22.5	22.5	22.5	22.5
18:3n-6	0	0	0	0	0
20:4n-6	0.1	0	0	0	0
n-3					
18:3n-3	15.0	15.2	15.1	15.2	15.1
18:4n-3	0.2	0.2	0.2	0.2	0.2
20:5n-3	0.8	0.8	0.8	0.8	0.8
22:6n-3	1.3	1.3	1.3	1.3	1.3
Total PUFA [3]	**40.4**	**40.6**	**40.4**	**40.6**	**40.4**
ALA [% DM] [4]	2.3	2.4	2.3	2.3	2.3
∑EPA+DHA [% DM] [4]	0.3	0.3	0.3	0.3	0.3

[1] Total SFA is the sum of saturated fatty acids; [2] Total MUFA is the sum of monounsaturated fatty acids; [3] Total PUFA is the sum of polyunsaturated fatty acids; [4] Calculated from the percentage data and the lipid content in the diets (in % DM), assuming 93% of total lipid to be fatty acids.

Sampling

Eleven acclimatized residual fish (three whole body, eight fillet and liver) were anesthetized, killed by a blow on the head and sampled for determining the initial status. Fish were starved for 72 hours for gastric emptying at the beginning and the end of the 52 day-feeding trial and bulk weighed for determination of growth performance parameters: Feed conversion ratio

(FCR): feed intake [g]/weight gain [g]; specific growth rate (SGR, [% d^{-1}]): [ln (final body weight) – ln (initial body weight)]/feeding day x 100; protein efficiency ratio (PER): weight gain [g]/protein intake [g]; protein retention efficiency (PRE): 100 x {[(final body protein x final body weight) – (initial body protein x initial body weight)]/protein intake}.

After the bulk weighing, final samples were taken. Both samplings (initial and final) were conducted following the same procedure. Three fish per tank were sacrificed as a pool sample for fatty acid and nutrient composition of whole body homogenate and stored at -20°C. Five fish per tank were used for sampling fillet, liver and spleen. Liver and spleen weights were measured to calculate hepatosomatic and spleen somatic index (Hepatosomatic index (HSI): 100 x (liver weight [g]/body weight [g]); Spleen somatic index (SSI): 100 x (spleen weight [g]/body weight [g]). One part of the liver was taken for measurement of Fads2a(d6) protein levels via ELISA and stored at -80°C. Another part of the liver was used for mRNA quantification via quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), preserved in RNA later to prevent RNA degradation and stored at -20°C. Residual liver and fillet tissue was pooled for fatty acid analysis and stored at -80°C and -20°C, respectively. Length and weight of all sampled fish was measured to calculate Fulton condition factor (K: 100 x (final body weight x final body length^{-3})). Whole body homogenate and fillet samples were freeze dried (alpha 1-4 LSC; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and homogenized with a grinding mill (Grindomix, Retsch, Haan, Germany).

Nutrient composition

Proximate nutrient analysis of ingredients, diets and whole body homogenate was performed at the laboratory of the Gesellschaft für Marine Aquakultur mbH according to EU guideline (EC) 152/2009 [34]. Dry matter was determined by drying samples (ED53 9010-0078; Binder GmbH, Tuttlingen, Germany) until constant mass. Same samples were incinerated in a muffle furnace (LE 6/11/P300; Nabertherm, Lilienthal, Germany) for crude ash analysis. Gross energy content was determined by bomb calorimetry (C 200; IKA-Werke GmbH & Co. KG, Staufen, Germany). Crude lipid content was analyzed according to the Soxhlet method (HYDROTHERM HT 6 and SOXTHERM 416; C. Gerhardt GmbH & Co. KG, Königswinter, Germany). Crude protein content was determined according to the methods of Kjeldahl (Scrubber K-415 and KjelFlex 360; BÜCHI Labortechnik GmbH, Essen, Germany and 877 Titrino plus; Deutsche METROHM GmbH & Co. KG, Filderstadt, Germany).

Fatty acid composition

Fatty acid composition of diets, liver, fillet and whole body samples was analyzed by LUFA-ITL GmbH, Kiel, Germany via gas chromatography (DGF, C-VI 10 a). Saponification with

methanolic NaOH and transmethylation of total lipids was used to prepare fatty acid methyl esters (FAME). For this, boron trifluoride and methanol were utilized (DGF, C-VI 11 a). GC via split-injection (column: CP-Sil 88 50 m x 0.25 mm x 0.2 µm or similar) separated FAME samples. FAME were detected by flame ionization detector (FID) using helium as a carrier gas and subsequently identified in comparison with certified fatty acid standard mixtures. Fatty acid composition was calculated as percent of FAME relative to total FAME.

RNA isolation and qRT-PCR

Total mRNA was extracted from liver samples of rainbow trout using the Innuprep RNA Mini Kit (Analytik Jena, Jena, Germany) according to the manufacturer's instructions. Before the total RNA isolation, the samples were homogenized in a TissueLyser II (Qiagen, Hilden, Germany). NanoDrop measurements (NanoDrop2000c; ThermoScientific, Waltham, MA, USA) were used to determine RNA concentration. Quantification of mRNA steady state levels of genes encoding proteins related to lipid metabolism was measured via qRT-PCR. The SensiFast SYBR No-ROX One-Step Kit (Bioline, London, UK) and a Rotor-Gene 6000 real-time PCR cycler (Corbett/Qiagen) were used for that. Primers and their respective annealing temperatures are shown in Table 1-3. Relative mRNA concentrations were calculated using a standard curve. The mRNA steady state levels of *fads2a(d6)* and *cpt1a/c* were normalized to the expression level of the housekeeping gene elongation factor 1 α (*ef1α*).

Table 1-3. Primer sequences (forward and reverse) and the respective annealing temperatures for mRNA measurements via qRT-PCR of samples from rainbow trout liver.

Genes	Primer sequences FW	Primer sequences Rv	Annealing temp. (°C)	Ref.
ef1α [a]	ACAAGCCCCTYCGTCTGCC	GCATCTCCACAGACTTSACCTCAG	61	[35]
fads2a(d6) [b]	GCTGGAGARGATGCCACGGA	TGCCAGCTCTCCAATCAGCA	61	[35]
cpt1a [c]	TCGATTTTCAAGGGTCTTCG	CACAACGATCAGCAAACTGG	55	[36]
cpt1c [c]	CGCTTCAAGAATGGGGTGAT	CAACCACCTGCTGTTTCTCA	59	[36]

[a] Elongation factor 1 α; [b] Fatty acyl desaturase 2a (delta-6); [c] Carnitine palmitoyl transferase 1.

Enzyme-Linked Immunosorbent Assay (ELISA)

Fads2a(d6) protein levels were determined using a Fish Fatty Acid Desaturase 2 ELISA Kit (MBS066226, MyBiosource Inc., San Diego, CA, USA; purchased from Biozol, Eching, Germany) following to the manufacturer's protocol. Liver samples of rainbow trout were diluted in phosphate buffered saline in a TissueLyser II (Qiagen, Hilden, Germany). After the centrifugation, standards and diluted samples were applied to the Microelisa multiwall plate. Samples were incubated and treated with horseradish peroxidase (HRP) conjugate reagent followed by multiple washings. Color intensity was determined at 450 nm using a Labsystems iEMS MF multiplate reader (MTX Lab Systems, Bradenton, FL, USA purchased from Thermo

Fisher Scientific, Darmstadt, Germany). The Fads2a(d6) protein concentration in liver samples was calculated via standard curve. Values were normalized to the total protein concentration.

Statistical analysis

The statistical software R (2017) was used to evaluate the data, including the packages gdata, gplots, lsmeans, multcomp, nlme and piecewiseSEM. Prior to the data evaluation, appropriate statistical models were defined: (1) statistical linear model for data per tank (body weight, DFI, FCR, SGR, PER, PRE, nutrient and fatty acid composition); (2) mixed models [37,38] with tank as random factor if values per fish (HSI, SSI, K, mRNA steady state levels, Fads2a(d6) protein level) were considered. The data were assumed to be normally distributed and to be homoscedastic. These assumptions are based on a graphical residual analysis. The statistical model included the treatment (C, BA, DA, EQ, G). Based on the model, a Pseudo R^2 was calculated [39] and multiple contrast tests (many-to-one) (e.g., see [40]) were conducted.

Results

Growth, performance and nutrient composition

All groups tripled their initial body weight within the experimental period of 52 days (Table 1-4). Final body weights did not differ significantly between dietary treatments. In addition, DFI, PER, PRE, SSI and HSI were not significantly affected by the supplementation of bioactive substances. Fish fed with DA had significantly ($p = 0.0497$) lower K values compared to fish fed the control diet. Furthermore, the nutrient composition analysis of whole body homogenates showed no significant differences between fish fed the diets with bioactive substances and fish fed the control diet (Table 1-5).

Tissue fatty acid composition

Generally, the fatty acid composition of the sampled tissue was not modified to a great extent by the dietary treatments. However, fish fed EQ showed significantly ($p = 0.037$) increased DHA levels in their whole body homogenate samples compared to the samples of fish fed the diet C. Furthermore, fish fed the diet G tended ($p = 0.059$) to have higher DHA levels than the fish fed with C (Table 1-6). In fillet samples, elevated DHA levels were found for EQ and G fed fish (Table 1-7). In addition, fillets of EQ fed fish showed significantly lower 18:3n-6 and 18:4n-3 levels compared to fish fed the control group ($p = 0.017$ and $p = 0.049$, respectively). Levels of SFA, MUFA and PUFA in whole body homogenate and fillet samples were not affected by the dietary treatments and remained in a similar range of concentrations as the initial samples (MUFA > PUFA > SFA). In contrast, livers of fish fed with EQ had significantly higher levels of the fatty acid 18:0 and total SFA ($p < 0.0001$ and $p = 0.021$, respectively)

(Table 1-8). Levels of MUFA and PUFA in livers, however, were not affected by the dietary treatments.

Table 1-4. Growth performance, feed intake, feed efficiency and biometric parameters of rainbow trout fed with the experimental diets C, BA, DA, EQ and G for 52 days.

	C	BA	DA	EQ	G
IBW [1]	83.4 ± 0.3	83.1 ± 0.3	83.2 ± 0.2	83.2 ± 0.0	83.6 ± 0.5
FBW [2]	255 ± 1.3	259 ± 3.4	259 ± 6.1	251 ± 6.3	257 ± 4.6
FCR [3]	0.98 ± 0.01	0.95 ± 0.02	0.95 ± 0.02	0.98 ± 0.02	0.98 ± 0.01
SGR [4]	2.15 ± 0.01	2.19 ± 0.03	2.19 ± 0.04	2.12 ± 0.05	2.16 ± 0.03
DFI [5]	2.10 ± 0.02	2.07 ± 0.03	2.08 ± 0.01	2.09 ± 0.01	2.11 ± 0.01
PER [6]	2.15 ± 0.02	2.24 ± 0.05	2.22 ± 0.05	2.17 ± 0.05	2.15 ± 0.03
PRE [7]	37.0 ± 0.9	38.0 ± 1.0	38.5 ± 0.4	37.8 ± 0.5	36.9 ± 0.2
SSI [8]	0.17 ± 0.08	0.15 ± 0.05	0.13 ± 0.05	0.15 ± 0.06	0.16 ± 0.05
HSI [9]	1.30 ± 0.16	1.29 ± 0.13	1.26 ± 0.14	1.29 ± 0.16	1.21 ± 0.19
K [10]	1.39 ± 0.08^{a}	1.34 ± 0.08	1.32 ± 0.08^{b}	1.38 ± 0.10	1.38 ± 0.08

[1] Average initial body weight [g]; [2] Average final body weight [g]; [3] Daily feed intake [% d^{-1}]; [4] Feed conversion ratio = feed intake [g]/weight gain [g]; [5] Specific growth rate [% d^{-1}] = [ln (final body weight) – ln (initial body weight)]/feeding day x 100; [6] Protein efficiency ratio = weight gain [g]/protein intake [g]; [7] Protein retention efficiency = 100 x {[(final body protein x final body weight) – (initial body protein x initial body weight)]/protein intake}; [8] Hepatosomatic index = 100 x (liver weight [g]/body weight [g]); [9] Spleen somatic index = 100 x (spleen weight [g]/body weight [g]); [10] Fulton condition factor = 100 x (final body weight x final body length^{-3}). Values (mean ± SD, IBW, FBW, FCR, SGR, DFI, PER, PRE: n = 3; HSI, SSI: n = 3 (5 fish/tank); K: n = 3 (8 fish/tank)) with different superscript letters within one row are significantly different ($p < 0.05$), values without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Materials and Methods.

Table 1-5. Nutrient composition of whole body homogenate (in % WW; gross energy in MJ/kg) of rainbow trout before the experiment (Initial) and after being fed the experimental diets C, BA, DA, EQ and G for 52 days.

in % WW	Initial	C	BA	DA	EQ	G
Moisture	72.6	67.8 ± 1.2	68.9 ± 0.3	68.3 ± 0.8	68.7 ± 0.3	68.0 ± 0.7
Crude ash	2.7	2.0 ± 0.1	2.2 ± 0.3	2.3 ± 0.1	2.3 ± 0.2	2.2 ± 0.2
Crude protein	16.2	16.9 ± 0.2	16.7 ± 0.1	17.0 ± 0.1	17.0 ± 0.2	16.8 ± 0.1
Crude lipid	8.6	13.3 ± 1.2	12.0 ± 0.2	12.4 ± 0.8	12.0 ± 0.5	12.8 ± 0.8
Gross energy [MJ/kg]	7.2	9.3 ± 0.5	8.8 ± 0.1	9.0 ± 0.3	8.9 ± 0.1	9.2 ± 0.3

Values (mean ± SD, n = 3) without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Methods. Initial data (n = 1, consisting of three fish) was not statistically analyzed.

Table 1-6. Fatty acid composition (in % of total fatty acid methyl ester (FAME)) of whole body homogenate of rainbow trout before the experiment (Initial) and after being fed the experimental diets C, BA, DA, EQ and G for 52 days.

[% of FAME]	Initial	C	BA	DA	EQ	G
14:0	1.4	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0
16:0	9.3	15.2 ± 0.1	15.2 ± 0.2	15.5 ± 0.5	14.8 ± 0.3	14.8 ± 0.5
18:0	2.7	3.9 ± 0.1	3.8 ± 0.2	3.9 ± 0.0	3.9 ± 0.1	3.8 ± 0.0
Total SFA [1]	**14.2**	**20.7 ± 0.2**	**20.7 ± 0.3**	**21.1 ± 0.6**	**20.4 ± 0.3**	**20.2 ± 0.6**
16:1	1.9	4.6 ± 0.3	4.4 ± 0.2	4.5 ± 0.2	4.0 ± 0.2[+]	4.4 ± 0.2
18:1	43.3	37.3 ± 0.5	37.3 ± 0.1	37.0 ± 0.5	36.8 ± 0.5	37.4 ± 0.7
Total MUFA [2]	**54.0**	**47.9 ± 0.7**	**47.6 ± 0.2**	**47.3 ± 0.5**	**47.1 ± 0.4**	**47.8 ± 0.9**
n-6						
18:2n-6	15.0	15.2 ± 0.4	15.5 ± 0.2	15.4 ± 0.1	15.6 ± 0.3	15.4 ± 0.3
18:3n-6	0.6	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.0
20:4n-6	0.7	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0
n-3						
18:3n-3	3.9	6.7 ± 0.3	6.6 ± 0.2	6.6 ± 0.0	6.7 ± 0.2	6.6 ± 0.3
18:4n-3	1.1	1.1 ± 0.0	1.1 ± 0.0	1.2 ± 0.0	1.1 ± 0.1	1.2 ± 0.1
20:5n-3	1.3	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
22:6n-3	6.2	3.9 ± 0.1[a]	3.9 ± 0.1	3.8 ± 0.1	4.2 ± 0.1[b]	4.2 ± 0.1[(b)]
Total PUFA [3]	**31.3**	**31.3 ± 0.7**	**31.4 ± 0.2**	**31.1 ± 0.5**	**31.9 ± 0.4**	**31.7 ± 0.9**

[1] Total SFA is the sum of saturated fatty acids; [2] Total MUFA is the sum of monounsaturated fatty acids; [3] Total PUFA is the sum of polyunsaturated fatty acids. Values (mean ± SD, n = 3, consisting of three fish each) with different superscript letters within one row are significantly different ($p < 0.05$), values without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Materials and Methods. [(b)] Brackets indicate a tendency towards a statistical difference ($p < 0.1$). Initial data (n = 1, consisting of three fish) was not statistically analyzed.

Hepatic fads2a(d6) mRNA steady state and Fads2a(d6) protein levels

Fish fed the diet EQ showed significantly ($p = 0.049$) decreased and fish fed the diet G tended ($p = 0.052$) to have lower hepatic *fads2a(d6)* mRNA steady state levels compared to fish fed the control group. *Cpt1a* and *cpt1c* levels were similar between the dietary treatments (Fig 1-1). Furthermore, hepatic Fads2a(d6) protein levels of rainbow trout were not affected by the dietary treatment (Fig 1-2).

Table 1-7. Fatty acid composition (in % of total fatty acid methyl ester (FAME)) of fillet of rainbow trout before the experiment (Initial) and after being fed the experimental diets C, BA, DA, EQ and G for 52 days.

[% of FAME]	Initial	C	BA	DA	EQ	G
14:0	1.2	1.2 ± 0.0	1.2 ± 0.1	1.1 ± 0.0	1.2 ± 0.0	1.2 ± 0.0
16:0	9.8	15.8 ± 0.2	15.6 ± 0.4	15.5 ± 0.0	15.8 ± 0.4	15.6 ± 0.0
18:0	3.0	3.8 ± 0.1	3.7 ± 0.1	3.8 ± 0.0	3.9 ± 0.1	3.9 ± 0.0
Total SFA [1]	**14.7**	**21.3 ± 0.1**	**21.1 ± 0.4**	**21.1 ± 0.3**	**21.4 ± 0.4**	**21.1 ± 0.1**
16:1	1.5	4.4 ± 0.1	4.4 ± 0.3	4.3 ± 0.3	4.5 ± 0.1	4.1 ± 0.0
18:1	41.1	35.1 ± 0.3	35.1 ± 0.3	35.1 ± 0.4	34.4 ± 0.2^{+}	34.7 ± 0.1
Total MUFA [2]	**50.7**	**45.0 ± 0.2**	**45.2 ± 0.0**	**45.0 ± 0.6**	**44.7 ± 0.3**	**44.3 ± 0.0**
n-6						
18:2n-6	14.2	15.0 ± 0.2	15.1 ± 0.1	15.1 ± 0.0	15.0 ± 0.2	15.3 ± 0.1
18:3n-6	0.5	0.6 ± 0.0^{a}	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0^{b}	0.6 ± 0.1
20:4n-6	0.2	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
n-3						
18:3n-3	3.4	6.5 ± 0.1	6.6 ± 0.1	6.6 ± 0.0	6.7 ± 0.0	6.6 ± 0.2
18:4n-3	0.9	1.4 ± 0.0^{a}	1.2 ± 0.0	1.2 ± 0.0	1.1 ± 0.1^{b}	1.3 ± 0.2
20:5n-3	1.6	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0
22:6n-3	10.2	5.7 ± 0.1	5.6 ± 0.1	5.8 ± 0.2	5.9 ± 0.2	6.1 ± 0.2
Total PUFA [3]	**34.4**	**33.4 ± 0.1**	**33.4 ± 0.3**	**33.4 ± 0.3**	**33.4 ± 0.5**	**34.1 ± 0.1**

[1] Total SFA is the sum of saturated fatty acids; [2] Total MUFA is the sum of monounsaturated fatty acids; [3] Total PUFA is the sum of polyunsaturated fatty acids. Values (mean ± SD, n = 3, consisting of fillets from five fish each) with different superscript letters within one row are significantly different ($p < 0.05$), values without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Materials and Methods. Initial data (n = 1, consisting of fillets from eight fish) was not statistically analyzed.

Table 1-8. Fatty acid composition (in % of total fatty acid methyl ester (FAME)) of liver of rainbow trout before the experiment (Initial) and after being fed the experimental diets C, BA, DA, EQ and G for 52 days.

[% of FAME]	Initial	C	BA	DA	EQ	G
14:0	0.7	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.1
16:0	15.9	14.6 ± 0.8	14.8 ± 0.1	14.4 ± 0.3	14.7 ± 0.3	14.7 ± 0.8
18:0	5.4	8.1 ± 0.2^{a}	7.8 ± 0.1	7.7 ± 0.4	10.2 ± 0.0^{b}	7.7 ± 0.7
Total SFA [1]	**23.3**	**24.2 ± 1.0^{a}**	**24.3 ± 0.1**	**23.4 ± 0.3**	**26.3 ± 0.2^{b}**	**23.8 ± 1.0**
16:1	0.8	2.7 ± 0.1	3.4 ± 0.3	3.1 ± 0.4	2.1 ± 0.1	3.1 ± 1.0
18:1	20.7	21.7 ± 2.6	23.0 ± 1.0	23.2 ± 2.6	16.3 ± 0.6	23.2 ± 6.2
Total MUFA [2]	**25.5**	**30.2 ± 2.7**	**32.7 ± 1.4**	**32.3 ± 3.1**	**24.7 ± 0.4**	**31.8 ± 8.0**
n-6						
18:2n-6	7.2	5.6 ± 0.5	5.3 ± 0.0	5.8 ± 0.4	5.5 ± 0.6	6.1 ± 0.2
18:3n-6	0.2	0.2 ± 0.0^{a}	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0^{b}
20:4n-6	4.7	5.8 ± 0.4^{a}	5.4 ± 0.1	5.7 ± 0.3	7.3 ± 0.4	3.5 ± 2.3^{b}
n-3						
18:3n-3	1.7	1.4 ± 0.2	1.1 ± 0.0	1.3 ± 0.2	1.5 ± 0.2	1.5 ± 0.1
18:4n-3	0.5	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
20:5n-3	3.9	2.2 ± 0.1	2.1 ± 0.0	1.9 ± 0.2	2.3 ± 0.3	2.2 ± 0.4
22:6n-3	29.5	25.2 ± 2.0	23.2 ± 1.2	23.7 ± 1.8	26.5 ± 0.9	23.8 ± 4.3
Total PUFA [3]	**50.8**	**45.4 ± 1.7**	**42.7 ± 1.3**	**43.8 ± 2.8**	**48.7 ± 0.6**	**44.1 ± 6.8**

[1] Total SFA is the sum of saturated fatty acids; [2] Total MUFA is the sum of monounsaturated fatty acids; [3] Total PUFA is the sum of polyunsaturated fatty acids. Values (mean ± SD, n = 3, consisting of livers from five fish each) with different superscript letters within one row are significantly different ($p < 0.05$), values without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Materials and Methods. Initial data (n = 1, consisting of eight livers) was not statistically analyzed.

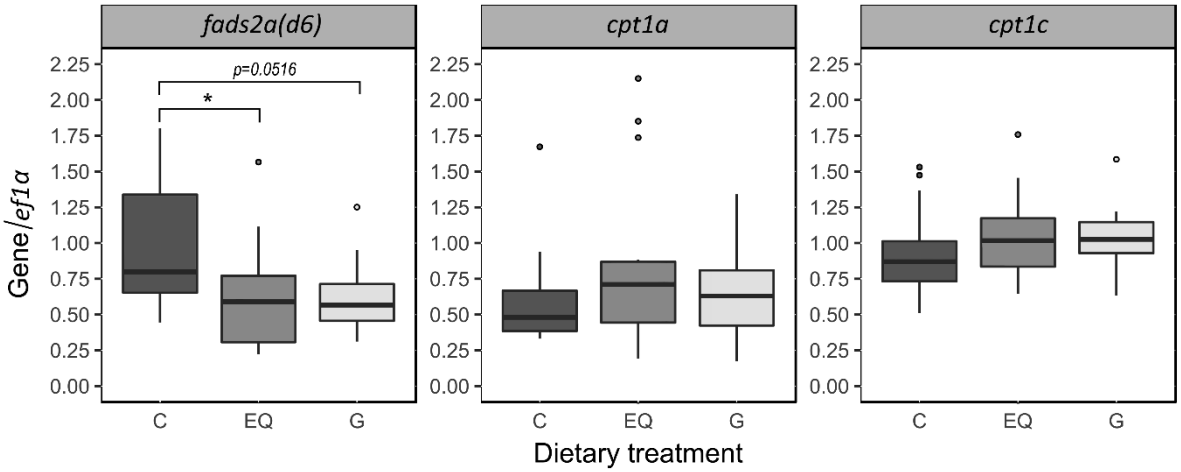

Fig. 1-1 Hepatic mRNA steady state levels. Rainbow trout were fed the experimental diets C, EQ and G for 52 days. Presented are boxplots of relative liver mRNA levels of fatty acyl desaturase 2a (delta-6) (*fads2a(d6)*) and carnitine palmitoyl transferase 1 (*cpt1*) a and c, respectively. Hepatic mRNA steady state levels were determined by qRT-PCR analysis and normalized to the housekeeping gene ef1α. Boxes represent values between the 25th and the 75th percentile; whiskers indicate 1.5 SD; medians are indicated by solid lines; outliers (above/below 1.5 SD) are indicated by solid circles. At the end of the experiment, 15 individuals per treatment were sampled in total (n = 15). Statistically significant differences between dietary treatments are represented by asterisks; $p < 0.05$ (*) based on the statistical models described in Materials and Methods.

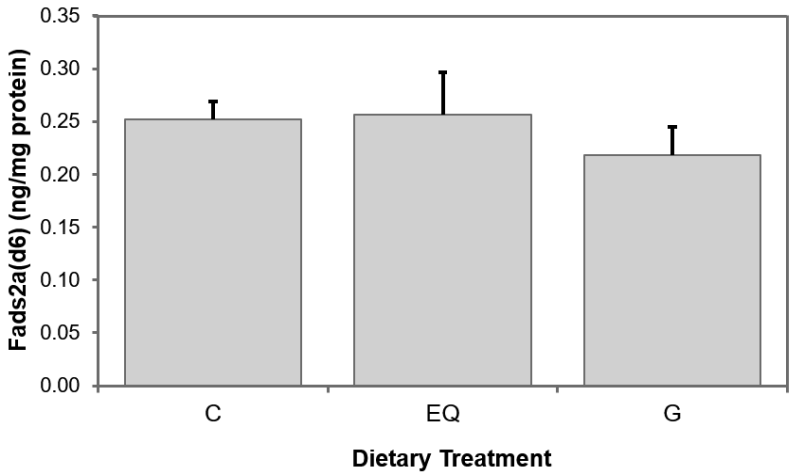

Fig. 1-2 Fatty acyl desaturase 2a (delta-6) (Fads2a(d6)) protein levels in liver samples. Rainbow trout were fed the experimental diets C, EQ and G for 52 days. Fads2a(d6) protein levels were determined by ELISA and were normalized to the total protein level (ng/mg protein). Bars represent the final expression values. At the end of the experiment, four individuals per treatment in total were sampled randomly (n = 4). Values were tested on significance based on the statistical models described in Material and Methods. No significant differences were found.

Discussion

In the present study, fish responded well to their dietary treatments and tripled their initial body weight within 52 days of feeding. The FCR (< 1.0) and SGR values (> 2.1 %/d) can be considered as reasonable since other studies reported similar, lower (SGR) and higher (FCR) values for rainbow trout of the same size [16,37,38]. No significant effect on growth performance was found between fish of any of the treatments in the present study. For EQ and G fed fish, these findings are in line with results in literature. Jourdehi et al. [39] also found no effect on growth in female beluga sturgeon fed with equol. Furthermore, Torno et al. [38] reported no negative impact on growth performance of rainbow trout fed genistein. In contrast, Crespillo et al. [40] found a reduction of body weights in rats fed a diet containing daidzein. A possible explanation for the lack of effect of the bioactive substances could be their dietary dosage utilized in the present study. Phytoestrogens have been shown to affect components of the growth hormone (GH)/insulin-like growth factor (IGF) axis in livers of rainbow trout [41]. The negative impact of genistein on the GH/IGF axis was enhanced when genistein was administered at a higher concentration [41]. The dose of 1.5 g/kg DM dietary genistein seemed to have no effect on the growth performance of rainbow trout in the present study and could thus be considered as a low dietary concentration. With regard to the observed effects in the present study, this assumption might also be true for equol, daidzein and biochanin A. Moreover, the feed conversion ratios were not affected in response to dietary bioactive compounds. Similar results were reported by Torno et al. [38] who fed genistein at 3.0 g/kg DM to rainbow trout. According to this study, dietary genistein did not affect feed conversion negatively but induced a significant reduction in feed intake. The authors assume that the bitterness of phytochemicals partly contributed to the decrease in feed intake. In the present study, dietary bioactive substances did not alter feed intake when daily feeding levels were set at 2.1% of biomass per day. A direct comparison is difficult, because fish in the present study were fed on a fixed daily feeding level that was below the reported feed intakes in the study of Torno et al. [38]. Furthermore, the dietary genistein concentration of 3.0 g/kg DM was twice the amount of the concentration used in the present study (1.5 g/kg DM). Thus, it seems that supplementation of 1.5 g/kg DM of these bioactive substances does not affect the palatability of the diets and does not influence feed intake negatively. Furthermore, PER and PRE values of fish as well as the nutrient composition of whole body homogenate samples were not affected by dietary bioactive substances in the present study. This is contrasting previous results of Torno et al. [38] who described significantly increased PER values and protein productive values (PPV, equals PRE) for rainbow trout fed with 3.0 g/kg DM of genistein. This, again, strongly supports the assumption that phytoestrogens and their metabolites might exhibit dose-dependent effects. In addition, Schiller Vestergren et al. [42] assumed that differences in the response to bioactive compounds could be due to varying physiological

parameters (species, fish size, gender, age) and environmental conditions (temperature, feed composition). The observations in the present study indicate that dietary equol, genistein, daidzein and biochanin A at 1.5 g/kg DM can be fed to rainbow trout without detrimental effects on growth and performance. Furthermore, any interactions due to the inclusion of soy protein concentrate into the diets are expected to be negligible. The overall content of isoflavones in soy is low with most of them being present as glucosinolates [43]. Glycosidic bond isoflavones need to be hydrolized in the intestine before absorption [43], requiring additional time for this process in the intestine [44]. Thus, we assumed that these substances might be mostly excreted and the amount of isoflavones being bioavailable can be considered as low.

The fatty acid composition of whole body homogenates and fillets was not affected to a great extent by the supplementation of bioactive substances in the present study. However, the DHA levels were increased in whole body homogenates and fillet samples of fish fed EQ and G in comparison to samples of fish fed the diet C. In contrast, EPA levels remained unaffected by the dietary treatments. For fish fed EQ, the increase in DHA could be possibly explained by its estrogenic potential [45]. In vertebrates, the synthesis of DHA occurs via the elongation (elongase 2) of 22:5n-3 to 24:5n-3 followed by a desaturation (delta-6-desaturation) to 24:6n-3 and a final peroxisomal β-oxidation [46]. The genes encoding for elongase 2 (*elovl2*) and the delta-6-desaturase (*fads2*) have been reported to be responsive to estrogen [26,28]. Estrogen application increased DHA in rats [26] and humans [47] by increasing the expression of *fads2*. Furthermore, equol showed a greater estrogenic potency than genistein, daidzein and biochanin A (equol > genistein > daidzein > biochanin A) in rainbow trout hepatocyte cultures [45]. In contrast to genistein and daidzein, equol is metabolically inert and can be readily absorbed [21]. This results inter alia in a higher bioavailability of equol in comparison to that of genistein [48]. Thus, the combination of the higher estrogenic potency and the increased bioavailability of equol putatively enhances the effect on the biosynthesis of DHA in comparison to the other bioactive substances in the present study. Simultaneously, the supplied amount of genistein, biochanin A and daidzein might be too low to develop estrogen-like effects. The significantly increased levels of the fatty acid 18:0 in the livers of fish fed EQ obtained in the present study support this hypothesis. For instance, an increase of 18:0 fatty acid has also been associated with increasing estrogen levels in rats [49]. Thus, it seems that dietary equol might have influenced the lipid metabolism via estrogen-like mechanisms. As the increase of both, DHA and 18:0 was only found for fish fed EQ it seems reasonable that other mechanisms led to the increased DHA levels in the G treatment. A factor possibly contributing to an increase in DHA are the antioxidant properties of genistein [23,50]. For example, genistein was found to protect cells from oxidative stress induced damage [51]. In the study of Hernandez-Montes et al. [51], genistein activated the transcription factor Nrf1 and thereby increased the activity and expression of its target gene glutathione peroxidase, an enzyme

involved in cellular defense mechanisms in endothelial cells. Thus, in the present study, genistein could have also increased the expression of glutathione peroxidase and thereby protected DHA from oxidation, leading to an indirect increase of DHA concentrations. However, bioactive substances could have further mechanisms of action to affect the DHA levels in fish. Trattner et al. [52] suggested that increased DHA levels in Atlantic salmon hepatocytes treated with the phytochemical sesamin might be a result of an increased peroxisomal β-oxidation. The peroxisomal β-oxidation is regulated inter alia by the transcription factor PPARα: PPARα binds to a peroxisome proliferator response element, thereby regulating the transcription of target genes centrally involved in the peroxisomal β-oxidation [53]. Ligands can bind to and activate PPARα. Genistein and daidzein have been found to be potent ligands for PPARα [31,32]. However, fish fed the diet G only tended to increase DHA levels in whole body homogenates and fish fed DA lacked an effect on tissue DHA in the present study. Thus, one could hypothesize that affecting the biosynthesis of DHA via PPARα is more complex and takes longer than directly influencing it via estrogen-like mechanisms. Furthermore, it could be possible that the dietary dosage of 1.5 g/kg DM of biochanin A and daidzein are too low to develop an effect either way.

The increase in DHA levels in whole body homogenates was not shown to the same extent in fillet samples of fish fed EQ and G diets. This could be partly explained by the very slow synthesis of DHA from ALA in rainbow trout [12]. Thus, it could be possible that the experimental duration in the present study was too short. It remains elusive whether fish fed EQ and G diets could have further increased their DHA fillet levels if the experimental period was longer. However, the effect of dietary equol and genistein on DHA tissue levels was only moderate. Thus, further investigations are needed to evaluate if and to what economically reasonable extent these bioactive substances can be included into rainbow trout diets to result in similar EPA and DHA levels that would be expected from fish fed a diet based on fish oil.

In the present study, hepatic mRNA steady state levels of *fads2a(d6)* were significantly lower in samples of fish fed EQ and tended to be lower in fish fed G compared to fish fed the diet C. Estrogen treatment enhanced the hepatic delta-6-desaturase expression and resulted in increased DHA levels in rats [26]. Thus, DHA concentrations in tissue samples were not well supported by the hepatic mRNA steady state levels. This effect has already been described in Atlantic salmon hepatocytes treated with the phytochemical sesamin [52]. In this study, DHA levels in hepatocytes were also significantly increased, although the gene expression of the gene encoding the delta-6-desaturase was down-regulated. A factor possibly contributing to the results in the present study could be the starvation time of fish before sampling. The half-life of genistein in rainbow trout was reported to be 13 h [54]. The authors suggest that the half-life of genistein in trout is similar to the values obtained in humans. Assuming that this is also applicable to other phytoestrogens, equol could have a half time of less than 10 h in trout

as it was reported for humans [21]. Therefore, it could be possible that by the time of sampling most of the genistein and equol has already been excreted by the fish. In addition, Schiller Vestergren et al. [55] assume that mRNA turnover responds to dietary changes. Thus, mRNA levels might fluctuate constantly. Another factor leading to the decreased hepatic *fads2a(d6)* mRNA steady state levels in the EQ and G fed groups could be the influence of LC-PUFA on gene expression. A negative feedback loop from n-3 LC-PUFA on the delta-6-desaturase mRNA expression has been found in zebrafish [56]. In the present study, DHA levels in whole body homogenate and fillet samples were highest in fish fed diets with EQ and G, respectively. Therefore, one could hypothesize that by the time of sampling, the increased DHA tissue levels in fish fed the EQ and G led to a negative feedback on *fads2a(d6)* mRNA expression. Interestingly, the Fads2a(d6) protein levels did not show significant differences between the dietary treatments. This mismatch of *fads2a(d6)* mRNA steady state and Fads2a(d6) protein levels has also been reported by Torno et al. [57] in a study with rainbow trout fed the phytochemical resveratrol. However, we cannot exclude putative effects of the bioactive substances on the delta-6-desaturase activity, as this factor was not analyzed in the present study. Furthermore, *cpt1a* and *cpt1c* mRNA steady state levels in the present study were not affected by the dietary treatments. Cpt1 is involved in the mitochondrial β-oxidation. The isoforms Cpt1a and Cpt1c are necessary for the uptake of long-chain fatty acids into the mitochondria [33]. Our results are contrasting the findings of Kim et al. [31], who reported that genistein induced the expression of Cpt1 via activating PPARα. Furthermore, these authors suggested that this effect was non-estrogenic and dose-dependent. Again, it seems that the dietary dosage of bioactive substances is important and needs to be considered when applied in rainbow trout.

Conclusion

The present study indicates that dietary equol, genistein, daidzein and biochanin A can be fed to rainbow trout at 1.5 g/kg DM without detrimental effects on growth and performance parameters. Furthermore, 1.5 g/kg DM of dietary equol increased DHA levels putatively by influencing the biosynthesis via estrogen-like mechanisms. However, this was not resembled in hepatic *fads2a(d6)* mRNA steady state levels and protein contents. In contrast, genistein seems to affect the biosynthesis of DHA either indirectly due to its antioxidative characteristics or via PPARα-mediated pathways. Biochanin A and daidzein did not affect the tissue fatty acid composition of rainbow trout. However, the increase in DHA levels due to dietary equol and genistein were only moderate. The dietary dosage of these substances might be an important factor influencing the extent of the effect on the biosynthesis of DHA. Thus, future studies should investigate dose-dependent effects of these bioactive substances on growth performance and LC-PUFA synthesis in rainbow trout.

Acknowledgements

We acknowledge financial support by Land Schleswig-Holstein within the funding programme Open Access Publikationsfonds. We would like to thank Dr. Mario Hasler and Jonas Schäler for their statistical advice and Dr. Hubert Wehage at the LUFA-ITL in Kiel, Germany, for the fatty acid analysis. Further, the authors would like to thank Bahne Christiansen, Julian Wessel, Sandra Nevermann and Petra Rettmann for their assistance during the experimental period and in the lab. In addition, we are grateful for the guidance given by Markus Griese, Michael Schlachter and all GMA colleagues for their help during sampling.

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CHAPTER 2

Are dietary genistein and equol potent enhancers of eicosapentaenoic acid and docosahexaenoic acid levels in rainbow trout (*Oncorhynchus mykiss*)?

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Modified version of the manuscript published in *Aquaculture research*, 2019, https://doi.org/10.1111/are.14097.

Abstract

Phytoestrogens are putatively able to enhance the biosynthesis of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), but have also been shown to affect fish growth dose-dependently. The aim of the present study was to identify a concentration for the phytoestrogen genistein and the phytoestrogen metabolite equol that further increases the endogenous biosynthesis of EPA and DHA without impairing fish growth. Juvenile rainbow trout (87.2 ± 0.3 g) were fed seven diets on a fixed ratio for eight weeks. A vegetable oil based diet served as a control diet and was supplemented with equol (EQ) and genistein (G), respectively at 0.1, 0.2 and 0.3% of feed dry matter (1, 2 and 3). Growth and nutrient composition of whole body homogenates were not affected by dietary treatments. EPA and DHA levels in liver, fillet and whole body samples were not significantly increased by EQ and G diets. Fish fed EQ diets showed dose-dependently increased liver weights and C18:0 liver levels, indicating estrogen-like effects at increased dietary dosages. In conclusion, the utilization of equol and genistein in plant oil based diets in order to enhance the biosynthesis of EPA and DHA seems not reasonable in rainbow trout.

Keywords: Rainbow trout; Phytochemicals; Biosynthesis; LC-PUFA; Aquafeed; Vegetable oils

Introduction

Generally, vegetable oils are used to fill the shortfall of fish and invertebrate oils in aquafeeds [1]. As long as the requirement levels of essential fatty acids are met, the total substitution of fish oil by vegetable oils is possible without detrimental effects on growth and health in some fish species, mainly salmonids (reviewed in [2]). This is due the capability of these fish species to endogenously synthesize omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA) from the precursor fatty acid α-linolenic acid (C18:3n-3, ALA) [3]. However, the efficiency of this biosynthesis pathway is limited and fish fed diets based on plant oils cannot maintain n-3 LC-PUFA tissue levels of fish fed fish oil based diets [4–6]. Especially, the content of eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) is reduced in fish fed a diet based on vegetable oils [4,7]. EPA and DHA are known for their health-promoting effects in humans [8–10]. Thus, the product quality of fish fed diets with vegetable oils is reduced for human consumption.

The utilization of dietary phytochemicals might be a promising strategy to increase EPA and DHA levels in rainbow trout fed with vegetable oils [11,12]. Recent investigations indicate that dietary genistein and equol at 0.15% of dry matter enhance tissue DHA levels in rainbow trout (*Oncorhynchus mykiss*) but only to a moderate extent [13]. Genistein and equol are phytochemicals, belonging to the group of phytoestrogens and their metabolites, respectively [14]. Genistein occurs naturally in soy (*Glycine max*) [15], whereas equol is a metabolite of the soy phytoestrogen daidzein [14]. Equol is not directly plant-derived but can be synthesized from its parent phytoestrogen daidzein by the intestinal bacteria in animals and humans [16,17]. Phytoestrogens such as genistein can bind to ligands that activate transcription factors (e. g. peroxisome proliferator-activated receptor α, PPARα) [18]. PPARα, in turn, regulates the transcription of target genes centrally involved in the peroxisomal β-oxidation [19] which is required for the synthesis of DHA. Furthermore, phytoestrogens have a similar structure as estradiol [20] and thus, show estrogenic properties in rainbow trout hepatocyte cultures [21]. The delta-6 desaturase is necessary for the synthesis of DHA out of EPA [22]. Estradiol application increases the expression of this desaturase in rats and thereby, increases DHA concentrations in livers and plasma of these animals [23]. Genistein and equol have been shown to exhibit antioxidant properties, estrogen-like effects and ligand-binding properties dependent on the dosage applied [24–26]. Therefore, a dose-dependent administration of genistein and equol might enhance the effect on the biosynthesis of DHA in rainbow trout found in our previous study [13]. However, when administered at higher dietary dosages the supplementation of phytochemicals is often associated with impaired fish growth [12,27,28] and reduced nutrient utilization [29].

The aim of the present study was to identify a concentration for genistein and equol that further increases the endogenous biosynthesis of EPA and DHA without impairing fish growth. Thus, a feeding trial was conducted with juvenile rainbow trout. A vegetable oil based diet was supplemented with equol and genistein at three differing dosages, respectively. Influences of the different dietary treatments on growth performance, fatty acid composition of liver, whole body and fillet, and nutrient composition of whole body were investigated.

Material and methods

Experimental setup

All-female rainbow trout juveniles (87.2 ± 0.3 g initial body weight) used in this experiment were obtained from a local fish farm (Forellenzucht Trostadt GbR, Reurieth, Germany). The feeding trial was conducted at the facilities of the Gesellschaft für Marine Aquakultur (GMA, Büsum, Germany). Fish were acclimatized to the experimental conditions in the recirculating aquaculture system (20 m^3, turnover rate 2.4 h^{-1}, technical oxygen supply). During this time, experimental animals were fed a commercial fish feed. A light/dark cycle of 14 h/10 h was adapted. The water purification system included a drum filter, biofilter, ultraviolet disinfection and protein skimmer with ozone. Water parameters were in range for rainbow trout. Oxygen (12.0 ± 0.2 mg/L O_2), temperature (14.8 ± 0.5°C) and pH (7.5) were monitored continuously via probes. NH_4^+ (0.3 ± 0.2 mg/L), NO_2^- (0.7 ± 0.3 mg/L) (Microquant test kits; Merck KGaA, Darmstadt, Germany) and salinity (4.2 ± 0.7 ‰) were measured daily. After two weeks acclimatization time, fish were weighed individually prior to the experimental start. A total of 420 fish were randomly distributed among 21 tanks of the recirculating aquaculture system and each tank (150 L) was stocked with 20 fish (1743.9 ± 6.7 g total biomass). The dietary treatments were randomly distributed in triplicates and fish were hand-fed once per day for eight weeks. All fish in one tank were bulk-weighed every 14 days to adjust daily feed supply at a daily feed intake level of 1.6% of biomass. The experiment was approved by the Ministry of Energy, Agriculture, the Environment, Nature and Digitalization (MELUND, Kiel, Germany; approved on 18 October 2017, project number V241-37421/2017) and carried out according to the national regulations for animal welfare (TierSchVersV) and the EU Directive 2010/63/EU for animal experiments.

Experimental diets

All seven diets were formulated based on the same feed ingredient composition (Table 2-1). The oil blend used as a dietary oil source consisted of palm fat, linseed, rapeseed and sunflower oil. This oil blend was designed to be above the dietary ALA requirement levels (requirement 0.7 - 1.0% of dry diet; see Table 2-2) of rainbow trout [30] as the aim of the study was to increase the biosynthesis of EPA and DHA. Further, dietary EPA and DHA levels were

restrictively supplied by only 15% of lean fish meal inclusion (recommendation 0.4 - 0.5% EPA + DHA of dry diet [30]).

Table 2-1. Ingredients and nutrient composition (in % of dry matter (DM)) of the experimental diets C, EQ1, EQ2, EQ3, G1, G2 and G3.

Diet	C	EQ1	EQ2	EQ3	G1	G2	G3
Ingredients [% DM]							
Fish meal [a]	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Blood meal [b]	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Feather meal [c]	5.5	5.5	5.5	5.5	5.5	5.5	5.5
Pea protein isolate [d]	12.0	12.0	12.0	12.0	12.0	12.0	12.0
Soy protein concentrate [e]	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Wheat gluten [f]	14.0	14.0	14.0	14.0	14.0	14.0	14.0
Gelatin [g]	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Wheat starch [f]	22.0	22.0	22.0	22.0	22.0	22.0	22.0
Oil sources	**11.5**	**11.5**	**11.5**	**11.5**	**11.5**	**11.5**	**11.5**
Rapeseed oil [h]	3.3	3.3	3.3	3.3	3.3	3.3	3.3
Linseed oil [i]	4.2	4.2	4.2	4.2	4.2	4.2	4.2
Palm fat [j]	2.2	2.2	2.2	2.2	2.2	2.2	2.2
Sunflower oil [h]	1.8	1.8	1.8	1.8	1.8	1.8	1.8
Vitamin Mineral premix [k]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Calcium hydrogen phosphate [l]	0.5	0.5	0.5	0.5	0.5	0.5	0.5
α-Cellulose [m]	2.8	2.8	2.8	2.8	2.8	2.8	2.8
Lysine [n]	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Methionine [o]	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Bentonite [p]	3.4	3.3	3.2	3.1	3.3	3.2	3.1
Genistein [q]	-	-	-	-	0.1	0.2	0.3
S-Equol [q]	-	0.1	0.2	0.3	-	-	-
Nutrient composition [% DM]							
Dry matter [in % of diet]	86.8	84.6	88.1	86.9	87.6	88.4	87.7
Crude protein	52.1	52.0	51.3	51.8	52.7	51.4	51.5
Crude lipid	16.2	16.0	16.1	16.1	15.6	15.9	16.0
Crude ash	6.2	6.1	6.0	5.9	5.1	5.0	4.7
Total COH [in % DM] [r]	25.5	25.9	26.7	26.2	26.7	27.6	27.8
Gross energy [MJ/kg DM]	23.0	23.1	23.3	23.3	23.4	23.0	23.1

[a] Lean fish meal "low ash", Bioceval GmbH & Co. KG, Cuxhaven, Germany; [b] Daka porcine bloodmeal, Daka Denmark A/S, Løsning, Denmark; [c] GePro Goldmehl, GePro Geflügel-Protein Vertriebsgesellschaft mbH & Co. KG, Diepholz, Germany; [d] Emsland-Stärke GmbH, Emlichheim, Germany; [e] Euroduna Food Ingredients GmbH, Barmstedt, Germany; [f] KRÖNER STÄRKE GmbH, Ibbenbüren, Germany; [g] Gustav Ehlert GmbH & Co. KG, Verl, Germany; [h] Food store, Büsum, Germany; [i] Makana Produktion und Vertrieb GmbH, Offenbach a.d. Queich, Germany; [j] DF 100 PT-PV; EFG Elbe Fetthandel GmbH, Geesthacht, Germany; [k] Emsland-Aller Aqua GmbH, Golßen, Germany; [l] JRS Pharma GmbH & Co. KG, Rosenberg, Germany; [m] Mikro-Technik GmbH & Co. KG, Bürgstadt am Main, Germany; [n] Biolys, Evonik Industries AG, Essen, Germany; [o] MetAmino, Evonik Industries AG, Essen, Germany; [p] Castiglioni Pes y Cía, Buenos Aires, Argentina; [q] Genistein: Cas No: 446-72-0, Equol: Cas No: 531-95-3, Xi'an Natural Field Bio-Technique Co., LTD, Xi'an Shaanxi, China; [r] Total COH = 100 – (crude protein + crude lipid + crude ash).

Three diets each were supplemented with equol and genistein, respectively with 0.1, 0.2 and 0.3% dry matter (DM) in substitution for the filler ingredient bentonite (EQ1, EQ2, EQ3, G1, G2 and G3). One diet without supplementation served as a control diet (C). The dietary fatty acid composition is shown in Table 2-2. The experimental diets were formulated according to the amino acid requirements of rainbow trout [30] based on the amino acid contents of the single ingredients. All diets were isoenergetic and isonitrogenous. A pelleting machine (Type 14U175, Amandus Kahl, Hamburg) was used to produce the pellets (4 mm diameter, 6 mm length).

Table 2-2. Fatty acid composition (in % of FAME*) of the experimental diets C, EQ1, EQ2, EQ3, G1, G2 and G3.

	C	EQ1	EQ2	EQ3	G1	G2	G3
14:0	0.5	0.5	0.5	0.5	0.5	0.5	0.5
16:0	18.8	18.5	18.5	18.4	18.5	18.5	18.5
18:0	3.5	3.5	3.5	3.5	3.5	3.6	3.5
Total SFA	**23.6**	**23.3**	**23.3**	**23.2**	**23.3**	**23.5**	**23.6**
16:1	0.3	0.3	0.3	0.3	0.3	0.4	0.3
18:1	29.3	29.1	29.3	29.1	29.1	29.1	29.0
Total MUFA	**32.1**	**32.2**	**32.2**	**32.1**	**32.2**	**32.3**	**32.1**
n-6							
18:2n-6	24.7	24.7	24.8	24.7	24.7	24.8	24.7
18:3n-6	tr †	tr	tr	tr	tr	tr	tr
20:4n-6	tr	tr	tr	tr	tr	tr	tr
n-3							
18:3n-3	16.7	17.2	17.1	17.2	17.1	17.0	17.1
18:4n-3	0.1	0.1	0.1	0.1	0.1	0.1	0.1
20:5n-3	0.5	0.5	0.5	0.5	0.5	0.5	0.5
22:6n-3	1.1	1.0	1.0	1.0	1.0	1.1	1.0
Total PUFA	**43.5**	**43.9**	**43.9**	**43.9**	**43.9**	**43.9**	**43.9**
ALA [% DM] [1]	2.5	2.6	2.6	2.6	2.5	2.5	2.5
EPA+DHA [% DM] [1]	0.2	0.2	0.2	0.2	0.2	0.2	0.2

[1] calculated from the percentage data and the lipid content in the diets (in % DM), assuming 93% of total lipid to be fatty acids; † tr = traces (values < 0.1); * FAME = Fatty acid methyl ester.

Sampling

For the determination of growth performance parameters, fish were bulk-weighed at the beginning and the end of the 56 days feeding trial after 72 hours starvation. Feed conversion ratio (FCR), specific growth rate (SGR), protein efficiency ratio (PER) and protein retention efficiency (PRE) were calculated following the equations in Table 2-3. After the final bulk-weighing and prior to the final sampling, fish were fed the experimental diets again for two days. This was done to maximize the quality of the samples, as physiological parameters might be very sensitive to an extended starvation time. The initial and the final sampling were conducted following the same procedure: Fish were starved for 24 hours before the sampling and were anesthetized and killed by a sharp blow on the head. After two weeks of acclimatization and before the experiment, residual fish were used to determine the initial

status. Five fish per tank (eight fish for initial sampling) were sacrificed for individual samples of liver, spleen and fillet. Liver and spleen samples were weighed to calculate the hepato- and spleen somatic index (HSI and SSI). Livers and fillets were pool-sampled for analysis of fatty acid composition and stored at -80°C and -20°C, respectively. Further, three fish per tank (initial: three fish) were killed, chopped and pool-sampled for nutrient composition and fatty acid composition of whole body homogenate. Samples were frozenand stored at -20°C. Afterwards, for the analyses, whole body homogenate and fillet samples were freeze-dried (alpha 1-4 LSC; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) to constant mass, followed byhomogenization with a cutting mill (Grindomix, Retsch, Haan, Germany). Length and weight was taken from all sampled fish to calculate Fulton condition factor (K, Table 2-3).

Nutrient composition

The analysis of the nutrient composition was performed at the laboratory of the Gesellschaft für Marine Aquakultur according to EU guideline (EC) 152/2009 [31]. Samples of diets and whole body homogenates were analyzed including content of DM, crude ash, crude protein, crude lipid and gross energy content. For determination of DM, samples were dried in a drying oven (ED53 9010-0078; Binder GmbH, Tuttlingen, Germany) at 103°C until constant mass (around 4.5 hours) followed by incineration in a muffle furnace (LE 6/11/P300; Nabertherm, Lilienthal, Germany) at 560°C for four hours to determine crude ash. Crude protein content was determined according to the method of Kjeldahl with a nitrogen to protein coefficient of 6.25 (KjelDigester K-449, Scrubber K-415, KjelFlex 360; BÜCHI Labortechnik GmbH, Essen, Germany and 877 Titrino plus; Deutsche METROHM GmbH & Co. KG, Filderstadt, Germany). Crude lipid content was analyzed according to the Soxhlet method using hydrolysis and extraction (HYDROTHERM HT 6 and SOXTHERM® 416, Multistat/SX PC; C. Gerhardt GmbH & Co. KG, Königswinter, Germany). The content of gross energy was analyzed using bomb calorimetry (C 200; IKA®-Werke GmbH & Co. KG, Staufen, Germany).

Fatty acid composition

Fatty acid composition of diets, liver, whole body homogenates and fillet samples was analyzed by LUFA-ITL GmbH, Kiel, Germany via gas chromatography (DGF, C-VI 10 a). Preparation of fatty acid methyl ester (FAME) was conducted by saponification with methanolic NaOH and transmethylation of total lipids. For that, boron trifluoride and methanol were utilized (DGF, C-VI 11 a). Afterwards, FAME samples were separated by split-injection (column: CP-Sil 88 50 m x0.25 mm x0.2 µm or similar) via gas chromatography. In addition, FAME were detected with a flame ionization detector (FID). For that, helium was used as a carrier gas. Further,

FAME were identified by comparison with certified standard mixtures. The distribution of fatty acids was calculated as percent of FAME in relation to total FAME.

Statistical analysis

All statistical data evaluations were performed using the statistical software R (2017), including the packages gdata, gplots, nlme, piecewiseSEM and multcomp. The data were considered as normally distributed based on a graphical residual analysis. Homoscedasticity or heteroscedasticity, respectively, was taken into account. The data evaluation started with the definition of appropriate statistical models: (1) statistical models based on general least squares [32] for FBW, FCR, SGR, PRE, PER, nutrient and fatty acid composition; (2) mixed models [33,34] with tank as random factor if values per fish (HSI, SSI, K) were considered. The statistical model included the additive (C, EQ, G) and the additive level (0, 1, 2, 3). Based on the model, a Pseudo-R^2 was calculated [35] and multiple contrast tests (e.g., see [36]) were conducted in order to compare different additive levels within one additive type and to the control group and further, the same additive levels across the additive type.

Results

Growth performance and nutrient composition

All fish more than doubled their weight during the eight weeks and showed no significant differences for values of final body weights and weight gain (%) (Table 2-3). Furthermore, FCR, SGR, PER and PRE values were not affected by the dietary treatments. However, the supplementation of dietary equol at 0.2 and 0.3% significantly increased HSI dose-dependently with fish fed EQ3 having the highest levels: C = EQ1 < EQ2 < EQ3. In addition, fish fed EQ2 and EQ3 had significantly higher HSI values than fish fed G2 and G3, respectively. In contrast, SSI values of EQ3 fed fish were significantly decreased compared to G3 fed fish. K values also differed significantly with fish of EQ2 having higher levels than fish of the dietary group G2. The nutrient composition of whole body homogenate samples was not affected by the dietary treatments (Table 2-4).

Table 2-3. Growth performance, feed intake, feed efficiency and biometric parameters of rainbow trout fed with the experimental diets C, EQ1, EQ2, EQ3, G1, G2 and G3 for eight weeks.

	C	EQ1	EQ2	EQ3	G1	G2	G3
IBW [1]	87.1 ± 0.36	87.5 ± 0.10	86.9 ± 0.16	87.5 ± 0.26	87.0 ± 0.44	87.1 ± 0.09	87.2 ± 0.17
FBW [2]	208.7 ± 1.1	207.3 ± 5.6	209.5 ± 1.0	205.9 ± 3.1	211.2 ± 3.0	208.5 ± 2.4	205.5 ± 3.9
FCR [3]	1.03 ± 0.01	1.04 ± 0.03	1.02 ± 0.01	1.06 ± 0.02	1.00 ± 0.02	1.01 ± 0.01	1.02 ± 0.01
SGR [4]	1.56 ± 0.01	1.54 ± 0.05	1.57 ± 0.01	1.53 ± 0.03	1.58 ± 0.02	1.56 ± 0.02	1.53 ± 0.03
PER [5]	2.16 ± 0.02	2.13 ± 0.06	2.16 ± 0.01	2.10 ± 0.05	2.16 ± 0.05	2.18 ± 0.02	2.16 ± 0.03
PRE [6]	39.5 ± 1.2	38.4 ± 2.8	38.4 ± 0.6	37.8 ± 1.6	39.7 ± 3.3	40.1 ± 1.7	37.7 ± 0.8
HSI [7]	1.63 ± 0.25^{a}	1.59 ± 0.24^{a}	1.93 ± 0.24^{bM}	2.25 ± 0.29^{cP}	1.67 ± 0.24	1.52 ± 0.19^{N}	1.62 ± 0.16^{Q}
SSI [8]	0.14 ± 0.09	0.10 ± 0.04	0.11 ± 0.04	0.09 ± 0.03^{P}	0.12 ± 0.05	0.15 ± 0.05	0.15 ± 0.04^{Q}
K [9]	1.37 ± 0.17	1.38 ± 0.14	1.40 ± 0.15 ^{M}	1.38 ± 0.09	1.35 ± 0.10	1.29 ± 0.9 ^{N}	1.33 ± 0.09

[1] Average initial body weight [g]; [2] Average final body weight [g]; [3] Feed conversion ratio = feed intake [g]/weight gain [g]; [4] Specific growth rate [% d^{-1}] = [ln (final body weight) – ln (initial body weight]/feeding day x 100; [5] Protein efficiency ratio = weight gain [g]/protein intake [g]; [6] Protein retention efficiency = 100 x {[(final body protein x final body weight) – (initial body protein x initial body weight)]/protein intake}; [7] Hepatosomatic index = 100 x (liver weight [g]/body weight [g]); [8] Spleen somatic index = 100 x (spleen weight [g]/body weight [g]); [9] Fulton condition factor = 100 x (final body weight x final body length^{-3}). Values (mean ± SD, IBW, FBW, FCR, SGR, PER, PRE: n = 3; HSI, SSI: n = 3 (5 fish/tank); K: n = 3 (8 fish/tank)) with different superscript letters within one row are significantly different ($p < 0.05$), values without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Material and methods. Tests are based on comparison of additive levels within one additive and the control (a, b, c: C and EQ diets) or comparisons of additives of the same level (EQ2 and G2: M, N; EQ3 and G3: P, Q).

Table 2-4. Nutrient composition of whole body homogenate (in % OM; gross energy in MJ/kg OM) of rainbow trout before the experiment (Initial) and after being fed the experimental diets C, EQ1, EQ2, EQ3, G1, G2 and G3 for eight weeks.

	Initial	C	EQ1	EQ2	EQ3	G1	G2	G3
Dry matter	27.1	30.2 ± 0.9	30.7 ± 0.8	30.6 ± 0.3	31.3 ± 0.6	30.8 ± 0.3	30.3 ± 0.3	29.9 ± 0.6
Crude ash	2.8	2.1 ± 0.1	2.2 ± 0.2	2.2 ± 0.1	2.1 ± 0.2	2.1 ± 0.2	2.0 ± 0.2	2.1 ± 0.3
Crude protein	16.4	17.5 ± 0.4	17.3 ± 0.6	17.2 ± 0.1	17.3 ± 0.2	17.6 ± 0.7	17.6 ± 0.4	17.0 ± 0.3
Crude lipid	7.9	10.5 ± 0.8	11.2 ± 0.6	11.2 ± 0.5	11.6 ± 0.8	10.9 ± 0.0	10.6 ± 0.5	10.6 ± 0.4
Gross energy [MJ/kg]	6.9	8.3 ± 0.4	8.5 ± 0.2	8.6 ± 0.1	8.7 ± 0.2	8.5 ± 0.1	8.4 ± 0.1	8.3 ± 0.2

Values (mean ± SD, n = 3) without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Material and methods. Initial data (n = 1, consisting of three fish) was not statistically analyzed.

Fatty acid composition

Generally, the fatty acid composition of sampled tissue was affected to a greater extend by the supplementation of dietary equol than by genistein. In whole body homogenates, fish fed the diets EQ3 and G3 showed the highest (non significant) EPA and DHA levels of all dietary treatments (Table 2-5).

In fillet samples, 18:0 levels were significantly higher for G1 fed fish compared to fish fed the control diet C (Table 2-6). In addition, fillets of fish fed diets containing equol had significantly increased levels of this fatty acid compared to fish fed the control diet. Furthermore, EPA fillet levels in EQ2 fed fish were significantly lower than in C and EQ1 fed fish. In contrast, DHA fillet levels were not altered by the dietary treatments.

Similar to the fillet samples, increased levels of 18:0 were found in the livers of fish fed diets with equol (C < EQ1 = EQ2 < EQ3) (Table 2-7). There was also a tendency ($p=0.052-0.069$) towards lower C18:1 levels in livers of fish fed with equol treatments when compared to fish fed the control diet. EPA levels were significantly higher in EQ1 fed fish compared to G1 fed fish. DHA levels did not differ significantly among treatments.

Table 2-5. Fatty acid composition (in % of total fatty acid methyl ester (FAME)) of whole body homogenate of rainbow trout before the experiment (Initial) and after being fed the experimental diets C, EQ1, EQ2, EQ3, G1, G2 and G3 for eight weeks.

	Initial	C	EQ1	EQ2	EQ3	G1	G2	G3
14:0	1.4	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.0
16:0	9.6	14.9 ± 0.9^{ab}	14.6 ± 0.2^{a}	14.7 ± 0.8^{ab}	14.0 ± 0.1^{b}	14.5 ± 0.8	14.5 ± 0.9	14.1 ± 0.3
18:0	2.7	4.0 ± 0.3	3.8 ± 0.0	4.0 ± 0.2	3.9 ± 0.1	3.9 ± 0.2	4.0 ± 0.3	3.7 ± 0.1
Total SFA	**14.4**	**20.7 ± 1.3**	**20.1 ± 0.3**	**20.3 ± 1.2**	**19.4 ± 0.2**	**20.1 ± 1.2**	**20.3 ± 1.3**	**19.5 ± 0.2**
16:1	1.8	4.1 ± 0.2^{ab}	4.2 ± 0.1^{a}	3.9 ± 0.3^{ab}	3.6 ± 0.2^{b}	4.0 ± 0.2	3.7 ± 0.1	3.8 ± 0.1
18:1	44.2	39.4 ± 1.1	38.2 ± 0.8	38.5 ± 1.6	37.6 ± 0.5	39.4 ± 1.3	40.0 ± 1.6	37.6 ± 0.8
Total MUFA	**54.9**	**49.0 ± 1.3**	**48.1 ± 1.2**	**48.4 ± 2.0**	**47.2 ± 0.6**	**49.3 ± 1.5**	**49.8 ± 1.8**	**47.1 ± 0.6**
n-6								
18:2n-6	14.9	16.1 ± 0.5	16.2 ± 0.2	16.1 ± 0.7	16.4 ± 0.2	15.9 ± 0.5	15.9 ± 0.8	16.5 ± 0.1
18:3n-6	0.4	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
20:4n-6	0.5	0.3 ± 0.1^{a}	0.3 ± 0.0^{a}	0.3 ± 0.0^{ab}	0.5 ± 0.1^{bP}	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0^{Q}
n-3								
18:3n-3	4.2	6.9 ± 0.8	7.1 ± 0.3	6.9 ± 0.8	7.6 ± 0.2	6.9 ± 0.6	6.7 ± 0.7	7.5 ± 0.0
18:4n-3	0.9	0.9 ± 0.1	0.9 ± 0.0	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
20:5n-3	1.2	0.5 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.0
22:6n-3	6.0	2.6 ± 0.7	3.3 ± 0.6	3.2 ± 1.0	3.9 ± 0.2	2.9 ± 0.9	2.6 ± 0.8	3.8 ± 0.4
Total PUFA	**30.5**	**30.1 ± 2.6**	**31.5 ± 1.4**	**31.0 ± 3.0**	**33.2 ± 0.6**	**30.5 ± 2.5**	**29.8 ± 2.7**	**33.3 ± 0.6**

Values (mean ± SD, n = 3, consisting of three fish each) with different superscript letters within one row are significantly different ($p < 0.05$), values without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Materials and methods. Tests are based on comparison of additive levels within one additive and the control (a, b, c: C and EQ diets) or comparisons of additives of the same level (EQ3 and G3: P, Q). Initial data (n = 1, consisting of three fish) was not statistically analyzed.

Table 2-6. Fatty acid composition (in % of total fatty acid methyl ester (FAME)) of fillet of rainbow trout before the experiment (Initial) and after being fed the experimental diets C, EQ1, EQ2, EQ3, G1, G2 and G3 for eight weeks.

	Initial	C	EQ1	EQ2	EQ3	G1	G2	G3
14:0	1.2	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0
16:0	10.2	14.9 ± 0.2	15.5 ± 0.2	15.0 ± 0.1	15.1 ± 0.4	14.8 ± 0.3	14.5 ± 0.3	14.5 ± 0.2
18:0	2.9	3.7 ± 0.0^{ax}	3.9 ± 0.1^{b}	3.9 ± 0.0^{b}	4.0 ± 0.1^{bP}	3.9 ± 0.0^{y}	3.8 ± 0.0^{xy}	3.8 ± 0.0^{xyQ}
Total SFA	**14.9**	**20.2 ± 0.2**	**21.0 ± 0.3**	**20.5 ± 0.1**	**20.6 ± 0.5**	**20.3 ± 0.4**	**19.9 ± 0.4**	**19.9 ± 0.2**
16:1	1.5	3.8 ± 0.3	3.8 ± 0.1	3.8 ± 0.0	3.6 ± 0.3	3.8 ± 0.1	3.5 ± 0.2	3.6 ± 0.1
18:1	39.1	35.1 ± 0.5	34.5 ± 0.3^{A}	35.2 ± 0.4	35.1 ± 0.2^{P}	35.8 ± 0.6^{B}	35.7 ± 0.5	35.7 ± 0.1^{Q}
Total MUFA	**48.9**	**44.0 ± 0.9**	**43.3 ± 0.3**	**44.4 ± 0.5**	**44.2 ± 0.5**	**44.7 ± 0.8**	**44.5 ± 0.4**	**44.6 ± 0.1**
n-6								
18:2n-6	14.0	15.9 ± 0.2	15.8 ± 0.2	15.8 ± 0.2	15.6 ± 0.2	15.9 ± 0.0	15.7 ± 0.1	16.0 ± 0.1
18:3n-6	0.3	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
20:4n-6	0.8	0.5 ± 0.1	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
n-3								
18:3n-3	4.1	7.5 ± 0.2	7.6 ± 0.2	7.3 ± 0.1	7.3 ± 0.2	7.5 ± 0.1	7.3 ± 0.1	7.4 ± 0.1
18:4n-3	0.7	1.0 ± 0.0^{ax}	1.0 ± 0.1^{abA}	0.9 ± 0.0^{ab}	0.8 ± 0.0^{b}	0.8 ± 0.0^{yB}	0.9 ± 0.0^{y}	0.9 ± 0.0^{xy}
20:5n-3	1.6	1.0 ± 0.1^{a}	0.9 ± 0.1^{a}	0.4 ± 0.1^{b}	0.6 ± 0.2^{ab}	0.6 ± 0.2	0.6 ± 0.0	0.6 ± 0.0
22:6n-3	11.9	6.2 ± 0.6	6.1 ± 0.2	6.1 ± 0.1	6.0 ± 0.2	5.5 ± 0.2	6.3 ± 0.2	6.0 ± 0.1
Total PUFA	**35.9**	**35.6 ± 1.0**	**35.4 ± 0.6**	**35.1 ± 0.4**	**34.8 ± 0.8**	**34.8 ± 0.5**	**35.4 ± 0.1**	**35.3 ± 0.3**

Values (mean ± SD, n = 3, consisting of fillets from five fish each) with different superscript letters within one row are significantly different ($p < 0.05$), values without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Materials and methods. Tests are based on comparison of additive levels within one additive and the control (a, b, c: C and EQ diets; x, y, z: C and G diets) or comparisons of additives of the same level (EQ1 and G1; EQ3 and G3: P, Q). Initial data (n = 1, consisting of eight fillets) was not statistically analyzed.

Table 2-7. Fatty acid composition (in % of total fatty acid methyl ester (FAME)) of liver of rainbow trout before the experiment (Initial) and after being fed the experimental diets C, EQ1, EQ2, EQ3, G1, G2 and G3 for eight weeks.

	Initial	C	EQ1	EQ2	EQ3	G1	G2	G3
14:0	0.5	1.4 ± 0.2^{a}	1.2 ± 0.0^{a}	1.1 ± 0.1^{abM}	0.8 ± 0.0^{bP}	1.3 ± 0.1	1.4 ± 0.0^{N}	1.3 ± 0.0^{Q}
16:0	16.4	21.1 ± 1.2^{a}	19.1 ± 0.6^{ab}	19.4 ± 0.2^{ab}	18.3 ± 0.8^{b}	19.7 ± 0.9	20.3 ± 0.3	19.5 ± 0.7
18:0	5.6	6.7 ± 0.1^{a}	8.7 ± 0.3^{bA}	9.3 ± 0.5^{bM}	10.4 ± 0.3^{cP}	7.2 ± 0.2^{B}	6.9 ± 0.2^{N}	6.8 ± 0.2^{Q}
Total SFA	**23.6**	**29.7 ± 1.4**	**29.5 ± 0.9**	**30.4 ± 0.5**	**30.3 ± 0.6$^{(P)}$**	**28.7 ± 0.9**	**29.0 ± 0.1**	**28.1 ± 0.1$^{(Q)}$**
16:1	0.6	4.5 ± 0.7	3.6 ± 0.2	3.7 ± 0.3	3.3 ± 0.4	3.5 ± 0.7	3.7 ± 0.2	3.4 ± 0.3
18:1	18.8	21.6 ± 1.4$^{(a)}$	18.3 ± 0.4$^{(b)}$	18.3 ± 0.8$^{(b)M}$	18.2 ± 0.8$^{(b)}$	21.1 ± 1.7	21.0 ± 0.8^{N}	19.9 ± 1.0
Total MUFA	**23.2**	**30.6 ± 2.2**	**27.2 ± 0.5**	**27.3 ± 0.6**	**26.9 ± 0.7**	**29.8 ± 2.0**	**29.4 ± 1.0**	**28.3 ± 1.0**
n-6								
18:2n-6	6.8	7.9 ± 0.4	7.5 ± 0.2	7.4 ± 0.2	7.1 ± 0.2^{P}	7.8 ± 0.0	8.3 ± 0.5	8.1 ± 0.3^{Q}
18:3n-6	0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:4n-6	4.4	2.6 ± 0.6	3.7 ± 0.4	3.1 ± 0.4	3.6 ± 0.4	2.6 ± 0.4	2.8 ± 0.2	3.0 ± 0.6
n-3								
18:3n-3	1.5	1.8 ± 0.2	1.6 ± 0.0	1.5 ± 0.0	1.4 ± 0.1	1.7 ± 0.1	1.9 ± 0.3	1.8 ± 0.1
18:4n-3	0.3	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
20:5n-3	3.3	1.8 ± 0.2	2.0 ± 0.0^{A}	1.7 ± 0.0	1.7 ± 0.1	1.6 ± 0.0^{B}	1.6 ± 0.2	1.9 ± 0.1
22:6n-3	32.8	18.8 ± 1.6	21.1 ± 1.0	20.7 ± 0.6	21.1 ± 0.9	20.5 ± 2.0	19.9 ± 1.3	21.5 ± 0.9
Total PUFA	**53.0**	**39.3 ± 2.6**	**42.9 ± 1.2**	**42.2 ± 0.7**	**42.5 ± 1.4**	**41.2 ± 2.6**	**41.4 ± 0.9**	**43.3 ± 2.0**

Values (mean ± SD, n = 3, consisting of livers from five fish each) with different superscript letters within one row are significantly different ($p < 0.05$), values without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Materials and methods. Tests are based on comparison of additive levels within one additive and the control (a, b, c: C and EQ diets) or comparisons of additives of the same level (EQ1 and G1: A, B; EQ2 and G2: M, N; EQ3 and G3: P, Q). Brackets indicate a tendency ($p < 0.1$) towards a statistical difference. Initial data (n = 1, consisting of eight livers) was not statistically analyzed.

Discussion

Effects of dietary equol and genistein on growth and EPA and DHA levels

In the present study, all fish more than doubled their initial body weight and showed similar final body weights among dietary treatments. This is in line with recent studies of Torno et al. [37] who fed genistein to rainbow trout at 0.3% without detrimental effects on growth performance of fish. Furthermore, own investigations revealed no negative effects of 0.15% dietary equol supplementation on growth of rainbow trout [13]. Thus, it seems that these findings can be confirmed in the present study and that dietary equol and genistein do not impair growth of rainbow trout when the dietary doses of 0.1 - 0.3% are fed with fixed feeding ratios of 1.6% of biomass/day. In accordance, FCR and SGR values did not differ significantly between the dietary treatments. The FCR and SGR values were in an acceptable range as other studies reported similar or higher (FCR) and lower (SGR) values for rainbow trout of the same size [6,37,38]. Moreover, the nutrient composition of whole body homogenates was not significantly altered by the dietary treatments. This is in accordance with findings of D'Souza et al. [39] who fed diets containing genistein to rainbow trout. Thus, it seems that dietary genistein and equol at 0.1 - 0.3% did not negatively affect the nutrient utilization. Furthermore, PER and PRE values were not affected in the present study. This is contrasting results of Torno et al. [37] who found increased PER and PPV (equals PRE) values for rainbow trout fed 0.3% of genistein. The discrepancies between the two studies might be related to differing daily feed intakes as the feed intake in the study of Torno et al. [37] was higher compared to the one in the present study (2.2% and 1.6% of biomass/day, respectively). By increasing the feeding level, the total amount of ingested bioactive substances increases and thus, the effects of these substances on protein retention could possibly be enhanced. Therefore, when comparing results of different studies using bioactive substances, it must be considered both, the dietary dosage and the feeding level. However, it seems that dietary genistein and equol can be included into rainbow trout feeds at levels of 0.1 - 0.3% without impairing growth performance and nutrient utilization when fed a fixed feeding ratio of 1.6% of biomass/day.

The evaluation of dietary equol and genistein at differing concentrations revealed no significant enhancement of EPA and DHA tissue contents in rainbow trout. EPA in fillet was significantly decreased in fish fed EQ2 and lower in fish fed EQ3 in comparison to fish fed the control diet. The application of the phytoestrogen sesamin also decreased EPA levels in Atlantic salmon hepatocytes, putatively due to an increased biosynthesis of DHA [40]. Bou et al. [41] showed that low dietary levels of DHA increase the conversion of EPA to DHA in Atlantic salmon. In contrast to these findings, DHA levels in fillet and liver samples of fish fed EQ2 and EQ3 were not increased in the present study. However, DHA levels were slightly increased (non significant) in whole body homogenates of these fish. A factor contributing to this result could

be the very slow synthesis efficiency of DHA in rainbow trout [3]. In addition to that, phytochemicals can affect the biosynthesis of DHA via different mechanisms, for example by increasing the expression of genes encoding proteins or by increasing the levels of proteins centrally involved in the biosynthesis [12,28,40]. These pathways putatively require additional time to further increase DHA tissue levels of fish. However, no significant effects of dietary application of equol and genistein on DHA tissue levels within the experimental duration were found in the present study. Thus, an extension of the experimental duration would have shown most likely only a marginal, if any, increase of DHA levels in fillets of fish.

Nevertheless, results of the present study are not in line with our initial hypothesis that higher dietary levels of bioactive substances might increase the effect on the biosynthesis of DHA. Own investigations revealed that 0.15% of dietary equol significantly increased and genistein tended to increase DHA levels of whole body homogenates in rainbow trout [13]. The difference between the dietary EPA and DHA concentration of the previous and the present study (EPA + DHA: 2.1% and 1.6% of FAME, respectively) could have partly contributed to these results. Low dietary long-chain polyunsaturated fatty acids levels increase the biosynthesis of EPA and DHA in rainbow trout [5,42]. However, it might be possible that the lower dietary EPA and DHA levels in the present study already challenged our rainbow trout. Thus, it seems that genistein and equol increase the efficiency of the EPA and DHA biosynthesis in rainbow trout only if the dietary levels of these fatty acids are higher than in the present study.

Effects of dietary equol on liver metabolism

In contrast to growth performance and the tissue contents of EPA and DHA, there was a dose-dependent effect of dietary equol on livers of rainbow trout. Liver weights and thus, HSI levels of rainbow trout were significantly increased and showed the highest values in fish fed the EQ3 diet. Similar results were found in livers of rainbow trout fed diets containing equol at levels of 0.2% and 0.3% of DM in our previous study [11]. These findings might be attributable to the estrogenic properties of equol due to its structural similarity to estradiol [20]. A dose-dependent increase of liver to body weight ratios due to estradiol application in rainbow trout is associated with the presence of vitellogenin [43]. Equol induces hepatic vitellogenin synthesis in Siberian sturgeon when administered via injection [44]. As vitellogenin synthesis can be induced already in immature fish [45], it is possible that dietary equol induced vitellogenin synthesis in the present study and thereby increased liver weights of rainbow trout. In addition to these results, there was a dose-dependent effect on C18:0 levels in liver samples and increased levels of this C18:0 in fillet samples of fish fed the EQ diets. The synthesis of C18:0 from C16:0 requires an elongation step mediated by the enzyme elongase 6 [46]. Elongase 6 is increased in ovariectomized rats treated with estradiol [47]. Further, overexpression of elongase 6

increases C18:0 in INS-1 cells (rat insulinoma cell line) [46] and in goat mammary cells [48]. Thus, it might be plausive to speculate that the estrogenic potential of equol is primarily responsible for the increase of C18:0 in fish fed EQ diets by increasing the expression of elongase 6. Furthermore, these effects seem to be more pronounced in response to increased dietary dosages of equol. Whether the increase in liver weight induced by dietary equol impairs health of rainbow trout needs further investigation (e.g. liver histology).

Overall, mainly the livers of rainbow trout seem to be affected by the dietary equol supplementation. Pelissero et al. [49] assume that dietary phytoestrogens possibly reach the liver, similar to estradiol, via the entero-hepatic circulation. The liver has the highest ER amounts among all tissues in rainbow trout [50]. In addition, equol can bind to estrogen receptors [51]. Furthermore, the fact that females have up to three times higher amounts of estrogen receptors than male rainbow trout [52] could have added to the effect of equol on the liver. Thus, equol might affect the liver via estrogen-like mechanisms. However, future studies should investigate whether (European) consumers accept phytoestrogens in rainbow trout diets that exhibit estrogen-like effects.

A factor contributing to the lack of activity of dietary genistein could be its lower estrogenic potency in rainbow trout hepatocyte cultures [21] and a lower bioavailability in comparison to equol [53].

Conclusion

In conclusion, present data indicate that dietary genistein and equol can be included into rainbow trout feeds at levels of 0.1 - 0.3% of dry matter of the diet without impairing growth performance and nutrient utilization when fish are fed 1.6% of biomass per day. Nevertheless, dietary equol putatively exhibited estrogen-like activity in livers of rainbow trout. Dietary equol and genistein were not able to increase the efficiency of the biosynthesis and the tissue levels of EPA and DHA in the present study. Therefore, the utilization of equol and genistein in plant oil based diets in order to enhance the biosynthesis of EPA and DHA seems not reasonable in rainbow trout.

Acknowledgements

This study was funded by the Federal Office for Agriculture and Food (grant number 2815NA044). We would like to thank Dr. Mario Hasler for his statistical advice and Dr. Hubert Wehage at the LUFA-ITL in Kiel, Germany, for the fatty acid analysis. Further, the authors would like to thank Adrian Schweiger, Carsten Rohwedder, Sandra Nevermann and Petra Rettmann for their assistance during the experimental period and in the lab. In addition, we are

grateful for the guidance given by Michael Schlachter and all GMA colleagues for their help during sampling.

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CHAPTER 3

Dietary *Buglossoides arvensis* oil as a potential candidate to substitute fish oil in rainbow trout diets

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Modified version published in *Lipids*, 2018, 53 (8): 809-823, https://doi.org/10.1002/lipd.12092.

Abstract

The utilization of vegetable oils in salmonid diets substantially decreased the body-content of omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) and thus, the product quality for human consumption. Therefore, new ingredients for aquaculture feeds are needed that maximize the deposition of the health promoting n-3 LC-PUFA. This study investigated *Buglossoides arvensis* (Ahiflower) oil, a plant oil rich in alpha-linolenic acid (18:3n-3, ALA) and stearidonic acid (18:4n-3, SDA), as a source of n-3 fatty acids in rainbow trout (*Oncorhynchus mykiss*) nutrition. Rainbow trout (87.4 ± 0.6 g) were fed for 56 days. The oils of the control diet (FV) were substituted by Ahiflower oil at 33, 66 and 100% (A33, A66, A100). Dietary Ahiflower oil increased the final body weights of fish. mRNA steady state levels of fatty acyl desaturase 2a (delta-6) (*fads2a(d6)*) and 2b (delta-5) (*fads2b(d5)*) as well as carnitine palmitoyl transferase 1 a (*cpt1a*) were not altered by dietary treatments. In contrast, *cpt1c* mRNA steady state levels were significantly downregulated in samples of fish fed A66 and A100. Significantly higher eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) levels were found in the liver and significantly higher EPA levels in the fillet of rainbow trout of A66 and A100 compared to FV. The content of DHA in fillets of fish fed Ahiflower oil was not significantly different to fish fed FV. Thus, high dietary amounts of Ahiflower oil can compensate for reduced dietary EPA and DHA levels.

Keywords: Rainbow trout, Ahiflower oil, Stearidonic acid, Oil replacement, Fatty acid metabolism, long-chain polyunsaturated fatty acids (LC-PUFA)

Introduction

Fish plays a crucial role in human nutrition. Besides its highly valuable protein, minerals and vitamins [1], fish provides important omega-3 (n-3) fatty acids such as eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) [1,2]. These fatty acids are known for a variety of beneficial effects in humans such as supporting optimal cell and tissue function as well as promoting health [3–5]. Western diets are rich in omega-6 (n-6) fatty acids and low in n-3 fatty acids [6]. High dietary intake of n-6 fatty acids can cause an imbalance in n-3:n-6 ratio in humans. This imbalance has been shown to increase the production of proinflammatory eicosanoids synthesized from arachidonic acid (20:4n-6) [7,8]. To achieve a balanced n-3:n-6 ratio, several health advisory organizations recommend the daily consumption of up to 650 mg EPA and DHA via two servings of fish per week [4].

Farmed rainbow trout (*Oncorhynchus mykiss*) receive n-3 long-chain polyunsaturated fatty acids (LC-PUFA) mainly via feeds traditionally based on fish oil as lipid source [9,10]. During the past few decades, dietary fish oil has been partially replaced by vegetable oils in fish diets [11,12]. However, vegetable oils are generally richer in n-6 PUFA than fish oil and devoid of n-3 LC-PUFA [13], leading to an unfavorable modification of the fatty acid composition of farmed fish [14–17]. This results in a reduced quality of rainbow trout for human consumption. Thus, new ingredients for aquaculture feeds that ensure the nutritional benefits of fish for humans are needed.

Rainbow trout are able to compensate lower dietary EPA and DHA levels via the endogenous fatty acid biosynthesis but only to a limited extend [18]. The n-3 LC-PUFA biosynthesis of fatty acid mainly takes place in the liver of fish [19]. Biosynthesis of n-3 LC-PUFA is initiated by the conversion of the precursor fatty acid alpha-linolenic acid (18:3n-3, ALA) to stearidonic acid (18:4n-3, SDA) catalyzed by the enzyme delta-6-desaturase. Furthermore, it requires a chain elongation step followed by delta-5-desaturation to form EPA. Synthesizing DHA from EPA involves, inter alia, a second delta-6-desaturation [20]. However, the efficiency of the endogenous n-3 LC-PUFA biosynthesis is limited [18]. Thus, bypassing the initial step (ALA to SDA) by providing dietary SDA may enhance the biosynthesis of EPA and DHA. Studies of humans [21,22], mice [23] and dogs [24] revealed that SDA is converted into EPA up to fourfold more efficiently than ALA, indicating SDA as a superior substrate for n-3 LC-PUFA synthesis. When applied in Atlantic salmon feed, dietary SDA affected tissue n-3 LC-PUFA contents positively. In Atlantic salmon parr, increased dietary SDA led to significantly higher amounts of SDA, EPA and DHA in red and white muscle tissues in comparison to feeding canola oil [25]. Similar results were obtained by feeding a plant oil rich in SDA (Echium oil) to seawater Atlantic salmon. In comparison to canola oil, feeding Echium oil resulted in higher contents of SDA and

EPA in whole body tissue [26]. In the same species, Echium oil upregulated delta-5-desaturase gene expression in the liver [27].

The plant oil with the naturally highest SDA levels known is Ahiflower oil [28]. It is extracted from the seeds of *Buglossoides arvensis.* Further, it is characterized by a favorable n-3:n-6 ratio due to low levels of linoleic acid (18:2n-6) and high amounts of ALA (Table 3-1). Moreover, it has a relatively neutral flavor which is described as “earthy […and] slightly nutty” [29]. This could be an advantage over plant oils with intensive flavoring like linseed oil, impairing feed intake in rainbow trout negatively when applied in higher dosages [30]. Thus, Ahiflower oil seems to be a promising candidate for fish oil replacement. Previously, this oil has been investigated in mice and humans. In mice, Ahiflower oil increased EPA and DHA levels in liver in comparison to controls fed flaxseed oil that is rich in ALA [28]. Lefort et al. [31] observed a dose-dependent increase of ALA and EPA levels in plasma of humans following dietary Ahiflower oil administration.

Table 3-1. Fatty acid composition (in mole%) of oil ingredients utilized in the experimental diets FV, A33, A66 and A100.

Fatty acid	Fish oil	Linseed oil	Palm oil	Rapeseed oil	Sunflower oil	Ahiflower oil
Total SFA	**16.5**	**10.4**	**81.8**	**7.1**	**10.7**	**7.0**
Total MUFA	**52.5**	**20.6**	**15.1**	**64.1**	**28.1**	**11.6**
n-6						
18:2n-6	13.8	15.7	3.0	19.3	60.5	12.0
18:3n-6	0.1	tr[§]	tr	tr	tr	5.3
n-3						
18:3n-3	6.1	52.9	tr	8.6	0.1	45.1
18:4n-3	0.8	tr	tr	tr	tr	18.4
20:5n-3	2.7	tr	tr	tr	tr	tr
22:6n-3	3.6	tr	tr	tr	tr	tr
Total PUFA	**30.2**	**68.6**	**3.0**	**27.9**	**60.6**	**80.8**

[§] tr = traces (values < 0.1)

To our knowledge, at the time of writing, there are no studies investigating Ahiflower oil in fish nutrition. Furthermore, new ingredients for aquafeeds are needed that maximize the quality of fish as a product for human consumption. Therefore, we aimed to evaluate the potential of Ahiflower oil as a source of n-3 fatty acids in rainbow trout nutrition, its effects on liver gene expression, and product quality for human nutrition. We were specifically interested to determine if high dietary levels of ALA and SDA would promote higher concentrations of EPA and DHA in tissues of rainbow trout. Thus, we substituted Ahiflower oil in a stepwise manner (0%, 33%, 66% and 100%) for an oil blend to determine an optimal dietary inclusion level for rainbow trout. EPA levels were significantly increased in the fillets of fish fed 66% and 100% of Ahiflower oil whereas DHA levels remained unaffected. Thus, high inclusion levels of dietary Ahiflower oil can compensate for reduced dietary levels of EPA and DHA, making Ahiflower oil a promising option to substitute fish oil in rainbow trout diets.

Materials and Methods

Experimental setup

The feeding trial was performed in a recirculating aquaculture system (RAS) at the Gesellschaft für Marine Aquakultur mbH (GMA, Büsum, Germany). Monosex female rainbow trout used in this experiment were obtained from a local fish farm (Forellenzucht Trostadt GbR, Germany). Before starting the experiment, fish were acclimatized to the experimental conditions and fed on a commercial fish feed (AllerGold, 3mm, AllerAqua) for two weeks. A total of 240 juvenile rainbow trout (87.4 ± 0.6 g initial body weight) were randomly distributed among twelve tanks of the RAS (20 m^3, turnover rate 2.4 h^{-1}, technical oxygen supply via oxygen cone). The water purification system comprised of a drum filter (mesh size 20µm), biofilter, ultraviolet (UV) disinfection and protein skimmer with ozone. Temperature (14.8 ± 0.5°C), oxygen (12.0 ± 0.2 mg/L O_2) and pH (7.5) were continuously monitored via probes. NH_4^+ (0.32 ± 0.25 mg/L), NO_2^- (0.69 ± 0.27 mg/L) (Microquant test kit for NH_4^+ and NO_2; Merck KGaA, Darmstadt, Germany) and salinity (4.2 ± 0.7 ‰) were measured daily. Each tank with a volume of 150 L was stocked with 20 fish (1747 ± 12 g). Fish were individually weighed (± 0.1 g) at the start of the experiment. A light/dark (14 h/10 h) cycle was adapted. Each diet was randomly distributed in triplicate and was hand fed once per day for 56 days. Each tank was bulk weighed every 14 days to adjust daily feed intake at a daily feed intake (DFI) level of 1.6% of biomass. The experiment was carried out according to the EU Directive 2010/63/EU for animal experiments and the national regulations for animal welfare (TierSchVersV). Furthermore, it was approved by the Ministry of Energy, Agriculture, the Environment, Nature and Digitalization (MELUND, Kiel, Germany; approved on 18 October 2017, project number V241-37421/2017).

Experimental diets

Fish were fed four diets in total. All diets were formulated based on the same feed ingredient composition, differing only in their oil sources (Table 3-2). A diet based on fish oil and a blend of vegetable oils (FV) served as a reference diet. For the experimental diets, Ahiflower oil was substituted for FV at levels of 33%, 66% and 100% (A33, A66 and A100). All diets were isonitrogenous and isoenergetic. Fatty acid composition of oil ingredients and final diets is shown in Table 3-1 and Table 3-3, respectively. Dietary content of EPA and DHA decreased with an increasing level of Ahiflower oil. Dietary content of amino acids was calculated based on the amino acid contents of single ingredients. The experimental diets were formulated according to the amino acid requirements of rainbow trout [13]. Pellets were produced with a pelleting machine (Type 14U175, Amandus Kahl, Hamburg) with 4 mm diameter and 6 mm length.

Table 3-2. Ingredients and nutrient composition (in % of dry matter (DM)) of the experimental diets FV, A33, A66 and A100.

Diet	FV	A33	A66	A100
Ingredients [in % DM]				
Fish meal [a]	15.0	15.0	15.0	15.0
Blood meal [b]	5.0	5.0	5.0	5.0
Feather meal [c]	5.5	5.5	5.5	5.5
Pea protein isolate [d]	12.0	12.0	12.0	12.0
Soy protein concentrate [e]	5.0	5.0	5.0	5.0
Wheat gluten [f]	14.0	14.0	14.0	14.0
Wheat starch [f]	22.0	22.0	22.0	22.0
Oil sources	***11.5***	***11.5***	***11.5***	***11.5***
Fish oil [g]	5.0	3.4	1.7	-
Rapeseed oil [h]	0.2	0.2	0.1	-
Linseed oil [i]	4.0	2.7	1.4	-
Palm fat [j]	0.5	0.3	0.2	-
Sunflower oil [h]	1.8	1.2	0.6	-
Ahiflower oil [k]	-	3.8	7.6	11.5
Gelatin [l]	1.5	1.5	1.5	1.5
Vitamin Mineral premix [m]	1.0	1.0	1.0	1.0
Calcium hydrogen phosphate [n]	0.5	0.5	0.5	0.5
α-Cellulose [o]	2.8	2.8	2.8	2.8
Lysine [p]	0.7	0.7	0.7	0.7
Methionine [q]	0.1	0.1	0.1	0.1
Bentonite [r]	3.4	3.4	3.4	3.4
Nutrient composition [% DM]				
Dry matter [in % of diet]	86.3	86.5	87.2	90.1
Crude protein	52.0	51.7	51.7	50.8
Crude lipid	16.1	15.9	16.0	15.8
Crude ash	6.2	6.2	6.2	6.1
Total COH [in % DM] [s]	25.7	26.2	26.1	27.3
Gross energy [MJ/kg DM]	23.1	23.1	23.1	22.8

[a] Lean fish meal "low ash", Bioceval GmbH & Co. KG, Cuxhaven, Germany; [b] Daka porcine bloodmeal, Daka Denmark A/S, Løsning, Denmark; [c] GePro Goldmehl, GePro Geflügel-Protein Vertriebsgesellschaft mbH & Co. KG, Diepholz, Germany; [d] Emsland-Stärke GmbH, Emlichheim, Germany; [e] Euroduna Food Ingredients GmbH, Barmstedt, Germany; [f] KRÖNER STÄRKE GmbH, Ibbenbüren, Germany; [g] Bioceval GmbH & Co. KG, Cuxhaven, Germany; [h] Food store, Büsum, Germany; [i] Makana Produktion und Vertrieb GmbH, Offenbach a.d. Queich, Germany; [j] DF 100 PT-PV; EFG Elbe Fetthandel GmbH, Geesthacht, Germany; [k] Nature's Crops International, Kensington, Canada; [l] Gustav Ehlert GmbH & Co. KG, Verl, Germany; [m] Emsland-Aller Aqua GmbH, Golßen, Germany; [n] JRS Pharma GmbH & Co. KG, Rosenberg, Germany; [o] Mikro-Technik GmbH & Co. KG, Bürgstadt am Main, Germany; [p] Biolys, Evonik Industries AG, Essen, Germany; [q] MetAmino, Evonik Industries AG, Essen, Germany; [r] Castiglioni Pes y Cía, Buenos Aires, Argentina; [s] Total COH = 100 – (crude protein + crude lipid + crude ash)

Table 3-3. Fatty acid composition (in mole%) of the experimental diets FV, A33, A66 and A100.

Fatty acid	FV	A33	A66	A100
14:0	1.2	1.0	0.6	0.4
16:0	13.1	11.8	10.4	9.0
18:0	3.4	23.0	2.7	2.2
Total SFA	**18.5**	**16.4**	**14.1**	**11.7**
16:1	1.3	1.0	0.7	0.3
18:1	27.8	22.6	17.6	12.3
Total MUFA	**33.9**	**27.3**	**21.6**	**15.2**
n-6				
18:2n-6	24.9	22.5	20.2	17.8
18:3n-6	0.0	1.4	2.6	4.0
20:4n-6	0.2	0.1	0.1	0.2
n-3				
18:3n-3	17.2	23.0	29.0	35.0
18:4n-3	0.3	4.7	9.1	13.7
20:5n-3	1.3	1.0	0.7	0.6
22:6n-3	2.0	1.7	1.3	1.0
Total PUFA	**47.0**	**55.4**	**63.5**	**72.3**
n-3:n-6 [1]	**0.8**	**1.3**	**1.8**	**2.3**

[1] n-3:n-6 is the ratio of ∑n-3 and ∑n-6 of n-3 and n-6 fatty acids, respectively, shown in this table.

Sample collection

Fish were bulk weighed at the beginning and the end of the 56-day-feeding trial after 72-h starvation to determine growth and performance parameters (Fig. 3-1). Specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and protein retention efficiency (PRE) were calculated following the equations in Table 3-5. After the final bulk weighing, fish were fed the experimental diets again for two days prior to final sampling, as physiological parameters might be very sensitive to an extended starvation time.

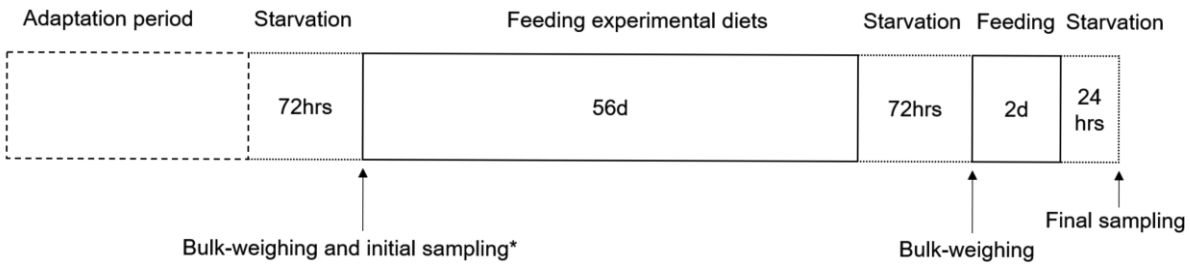

Fig. 3-1Timeline of the experimental period and sample collection. *Initial samples were taken from fish continuously fed the commercial diet of the adaptation period.

Both samplings (initial and final) were performed following the same procedure: Fish were deprived of feed 24 h before the sampling and were anesthetized and killed by a blow on the head. For the determination of the initial status, acclimated residual fish were used. Three fish per tank (pool sample) were sacrificed for nutrient composition of whole body homogenate and

stored at -20°C. Furthermore, five fish per tank (initial sampling: eight fish) were used for individual samples of liver, spleen, and fillet. Weight of liver and spleen was measured to calculate hepatosomatic and spleen somatic index (HSI and SSI). One part of the liver was used for mRNA quantification via quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). For that, samples were placed immediately into RNA later to prevent RNA degradation and stored on ice during sampling of each tank. In addition, these samples were incubated at 4°C overnight, followed by a storage at -20°C. Residual liver and fillet tissue was pool sampled for fatty acid analysis and stored at -80°C and -20°C, respectively. Length and weight were measured for all sampled fish to calculate the Fulton condition factor (K, Table 3-5). Fillet and whole body homogenate samples were freeze dried (alpha 1-4 LSC; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and homogenized using a cutting mill (Grindomix, Retsch, Haan, Germany).

Nutrient composition

Proximate nutrient analysis of diets and whole body homogenate included dry matter (DM), crude protein, crude lipid and crude ash. It was performed at the laboratory of the Gesellschaft für Marine Aquakultur according to EU guideline (EC) 152/2009 [32]. Dry matter was determined by drying the samples at 103°C in a drying oven (ED53 9010-0078; Binder GmbH, Tuttlingen, Germany) until constant mass. Afterwards, same samples were incinerated for ‘ 4 h at 560°C in a muffle furnace (LE 6/11/P300; Nabertherm, Lilienthal, Germany) to determine crude ash. Crude protein content was analyzed according to the methods of Kjeldahl via digestion (KjelDigester K-449 and Scrubber K-415; BÜCHI Labortechnik GmbH, Essen, Germany), distillation (KjelFlex 360; BÜCHI Labortechnik GmbH) and titration (877 Titrino plus; Deutsche METROHM GmbH & Co. KG, Filderstadt, Germany) using a nitrogen to protein coefficient of 6.25. Crude lipid content was determined according to the Soxhlet method using hydrolysis with hydrochloric acid (HYDROTHERM HT 6; C. Gerhardt GmbH & Co. KG, Königswinter, Germany) and extraction with petroleum ether (SOXTHERM 416, Multistat/SX PC; C. Gerhardt GmbH & Co. KG, Königswinter, Germany). Gross energy content was determined by bomb calorimetry (C 200; IKA-Werke GmbH & Co. KG, Staufen, Germany), calibrated with benzoic acid.

Fatty acid composition

Fatty acid composition was analyzed by LUFA-ITL GmbH, Kiel, Germany. Fatty acid composition of oils, diets, liver, and fillet samples was determined by gas chromatography (GC) (DGF, C-VI 10 a). Fatty acid methyl ester (FAME) were prepared by saponification with methanolic NaOH and transmethylation of total lipids using boron trifluoride and methanol (DGF, C-VI 11 a). Further, FAME samples were separated by GC via split-injection (column:

CP-Sil 88 50 m x 0.25 mm x 0.2 µm or similar) and detected with flame ionization detector (FID). Helium was used as carrier gas. FAME were identified by comparison with a certified standard mix (18919-1AMP Supelco, F.A.M.E. Mix, Sigma-Aldrich). The resulting FAME values were converted to moles and reported as mole% of total fatty acids.

RNA isolation and qRT-PCR

Approximately 15 mg of RNA later stabilized liver tissue of rainbow trout was homogenized in a TissueLyser II (Qiagen, Hilden, Germany) prior to total RNA isolation. Total RNA was extracted from liver samples using the Innuprep RNA Mini Kit (Analytik Jena, Jena, Germany) according to the manufacturer's protocol. RNA concentration and purity were determined by measuring the absorbance at ratios of A260/280 and 260/230 via NanoDrop measurements (NanoDrop2000c; ThermoScientific, Waltham, MA, USA). mRNA steady state levels of selected target genes were quantified via qRT-PCR measurements using the SensiFast SYBR No-ROX One-Step Kit (Bioline, London, UK) and a Rotor-Gene 6000 real-time PCR cycler (Corbett/Qiagen). Eigtheen microliters of a 1000-fold-diluted RNA sample were added to 27 µl Master Mix. The 27 µl Master Mix consisted of 2.48 µl DEPC treated water, 0.40 µl reverse transcriptase, 0.90 µl RNase inhibitor, 22.5 µl 2x SensiFAST-Mix and 0.36 µl of each primer. The analysis was done in duplicate using 20 µl of this mixture each. The cycling conditions were as follows: at 49°C for 30 min, at 95°C for 10 min, 40 cycles of 95°C for 15 s, annealing temperature of the primer pair for 30 s and 72°C for 30 s, followed by a melt curve analysis from 60 to 99°C of 1°C increments. Primer sequences and the respective annealing temperatures are shown in Table 3-4.

Table 3-4. Primer sequences (forward and reverse) and the respective annealing temperatures for mRNA measurements via qRT-PCR of samples from rainbow trout liver.

Gene	Primer	Sequence 5'→ 3'	Annealing Temperature (°C)
ef1α [a,*]	F	ACAAGCCCCTYCGTCTGCC	61
ef1α [a,*]	R	GCATCTCCACAGACTTSACCTCAG	61
fads2b(d5) [b,§]	F	GGACATTTCCTGGATGTTTTTGAT	57
fads2b(d5) [b,§]	R	TCTGACAAGGATTAAAACAATTATA	57
fads2a(d6) [c,*]	F	GCTGGAGARGATGCCACGGA	61
fads2a(d6) [c,*]	R	TGCCAGCTCTCCAATCAGCA	61
cpt1a [d,#]	F	TCGATTTTCAAGGGTCTTCG	55
cpt1a [d,#]	R	CACAACGATCAGCAAACTGG	55
cpt1c [d,#]	F	CGCTTCAAGAATGGGGTGAT	59
cpt1c [d,#]	R	CAACCACCTGCTGTTTCTCA	59

[a] *ef1α*: Elongation factor 1 α; [b] *fads2b(d5)*: fatty acyl desaturase 2b (delta-5) [c] *fads2a(d6)*: fatty acyl desaturase 2a (delta-6); [d] *cpt1*: Carnitine palmitoyl transferase 1; * Geay et al. [70]; § Gregory [71]; # Kolditz et al. [72].

A standard curve was used to calculate relative mRNA concentrations. The mRNA steady state levels of genes encoding proteins related to lipid metabolism were normalized to the

expression level of the housekeeping gene elongation factor 1 alpha (*ef1α*). *Ef1α* was previously used and suggested as a gene reference in salmonids [33].

Statistical analysis

The statistical software R (2017) was used to evaluate the data, including the packages gdata, gplots, nlme, piecewiseSEM and multcomp. The data evaluation started with the definition of appropriate statistical models: (1) statistical models based on general least squares [34] for final body weight, DFI, FCR, SGR, PER, PRE, nutrient and fatty acid composition and (2) mixed models [35,36] with tank as random factor if values per fish (HSI, SSI, K, *fads2a(d6)*, *fads2b(d5)*, *cpt1a*, *cpt1c*) were considered. The data were considered as normally distributed by a graphical residual analysis. Homoscedasticity or heteroscedasticity, respectively, was taken into account. Based on the model, a Pseudo R^2 was calculated [37] and multiple contrast tests (e.g., see [38]) were conducted to compare the dietary treatments. Furthermore, regression analysis was performed by fitting a linear model to fillet and diet fatty acid composition to show a potential relationship.

Results

Dietary Ahiflower oil increases final body weight

All groups more than doubled their initial body weight within the experimental period (Table 3-5). The final body weight was higher in all groups fed Ahiflower oil and significantly higher in A100 ($p < 0.05$) compared to FV. Furthermore, this group exhibited the lowest FCR and highest SGR values among treatments ($p > 0.05$). SSI was dose-dependently increased with significantly higher ($p < 0.05$) values in A66 and A100. DFI, PER, PRE, HSI and K were not significantly affected by the different dietary treatments.

Nutrient composition of whole body homogenates is not affected by dietary treatment

The analysis of nutrient composition of whole body homogenates showed no significant differences between groups in terms of crude ash, crude protein, crude lipid and gross energy (Table 3-6).

Table 3-5. Growth performance, feed intake, feed efficiency and biometric parameters of rainbow trout fed with the experimental diets FV, A33, A66 and A100 for 56 days.

	FV	A33	A66	A100
Initial weight [1]	87.1 ± 0.2	87.4 ± 0.9	87.7 ± 0.6	87.1 ± 0.2
Final weight [2]	210.3 ± 4.0^{a}	216.4 ± 1.0^{ab}	213.2 ± 2.0^{ab}	218.5 ± 2.2^{b}
DFI [3]	1.60 ± 0.01	1.60 ± 0.01	1.59 ± 0.01	1.59 ± 0.01
FCR [4]	1.02 ± 0.03	0.99 ± 0.01	1.00 ± 0.01	0.97 ± 0.02
SGR [5]	1.57 ± 0.03	1.62 ± 0.01	1.59 ± 0.03	1.64 ± 0.02
PER [6]	2.20 ± 0.06	2.27 ± 0.03	2.21 ± 0.03	2.26 ± 0.04
PRE [7]	39.1 ± 0.7	44.4 ± 4.7	37.8 ± 0.9	38.5 ± 2.1
HSI [8]	1.56 ± 0.21	1.52 ± 0.22	1.52 ± 0.17	1.53 ± 0.18
SSI [9]	0.12 ± 0.03^{a}	0.17 ± 0.09^{ab}	0.18 ± 0.05^{b}	0.21 ± 0.07^{b}
K [10]	1.38 ± 0.13	1.35 ± 0.09	1.39 ± 0.09	1.39 ± 0.11

[1] Average initial body weight [g]; [2] Average final body weight [g]; [3] Daily feed intake [% d^{-1}]; [4] Feed conversion ratio = feed intake [g]/weight gain [g]; [5] Specific growth rate [% d^{-1}] = [ln (final body weight) – ln (initial body weight)]/feeding day x 100; [6] Protein efficiency ratio = weight gain [g]/protein intake [g]; [7] Protein retention efficiency = 100 x {[(final body protein x final body weight) – (initial body protein x initial body weight)]/protein intake}; [8] Hepatosomatic index = 100 x (liver weight [g]/body weight [g]); [9] Spleen somatic index = 100 x (spleen weight [g]/body weight [g]); [10] Fulton condition factor = 100 x (final body weight x final body length^{3}). Values (mean ± SD, Initial weight, Final weight, DFI, FCR, SGR, PER, PRE: n = 3; HSI, SSI: n = 3 (5 fish/tank); K: n = 3 (8 fish/tank)) with different superscript letters within one row are significantly different ($p < 0.05$), values without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Materials and Methods.

Table 3-6. Nutrient composition of whole body homogenate (in % OM; gross energy in MJ/kg OM) of rainbow trout before the experiment (Initial) and after being fed the experimental diets FV, A33, A66 and A100 (mean ± SD, n = 3) for 56 days.

[in % OM]	Initial	FV	A33	A66	A100
Dry matter	27.1	30.8 ± 0.3	31.5 ± 2.1	30.6 ± 0.4	30.0 ± 0.4
Crude ash	2.8	2.1 ± 0.0	2.1 ± 0.0	2.1 ± 0.2	2.2 ± 0.2
Crude protein	16.4	17.2 ± 0.1	18.1 ± 1.1	16.8 ± 0.3	16.8 ± 0.5
Crude lipid	7.9	11.4 ± 0.2	11.3 ± 1.0	11.7 ± 0.4	11.0 ± 0.5
Gross energy [MJ/kg]	6.9	8.6 ± 0.1	8.7 ± 0.6	8.6 ± 0.2	8.3 ± 0.3

Values (mean ± SD, n = 3) without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Methods.

Dietary Ahiflower oil substitution dose-dependently alters fatty acid composition of fillet

Fatty acid composition of fillets dose-dependently altered due to the substitution of Ahiflower oil for FV (Table 3-7). Contents of fatty acids (in mole%) in the experimental groups A66 and A100 differed from FV to a greater extent than values of A33. A100 resulted in the highest ($p < 0.05$) fillet PUFA content of all treatments. A100 exhibited significantly ($p < 0.05$) higher levels of ALA and SDA (two- and sixfold, respectively) compared to FV. Consequently, the n-3:n-6 ratio increased significantly ($p < 0.05$) in a stepwise manner from FV to A100. In addition, EPA levels of fish fed with the diets A66 and A100 were identical and significantly ($p < 0.05$) higher than in fillets of fish fed with the diet FV. In contrast, increasing dietary Ahiflower oil levels led to lower ($p > 0.05$) DHA and significantly ($p < 0.05$) lower monounsaturated fatty acids and linoleic acid (18:2n-6) contents. Simultaneously, content of

γ-linolenic acid (18:3n-6) increased significantly from FV to A100. A66 and A100 also showed significantly lower saturated fatty acids levels than FV.

Table 3-7. Fatty acid composition (in mole%) of fillet of rainbow trout fed the experimental diets FV, A33, A66 and A100 for 56 days.

Fatty acid	Initial	FV	A33	A66	A100
14:0	1.5	1.7 ± 0.1^{a}	1.6 ± 0.1^{a}	1.4 ± 0.1^{b}	1.3 ± 0.0^{b}
16:0	11.3	15.8 ± 0.3^{a}	15.9 ± 0.1^{a}	15.1 ± 0.3^{ab}	15.0 ± 0.2^{b}
18:0	2.9	3.7 ± 0.1	3.7 ± 0.1	3.6 ± 0.2	3.7 ± 0.0
Total SFA	**16.3**	**21.8 ± 0.4^{a}**	**21.8 ± 0.2^{a}**	**20.5 ± 0.4^{b}**	**20.2 ± 0.2^{b}**
16:1	1.7	4.3 ± 0.1	4.2 ± 0.2	3.8 ± 0.2	3.7 ± 0.1
18:1	39.6	33.6 ± 0.6^{a}	30.5 ± 0.7^{b}	28.8 ± 0.3^{b}	26.0 ± 0.7^{c}
Total MUFA	**48.7**	**43.2 ± 0.8^{a}**	**39.4 ± 0.7^{b}**	**36.9 ± 0.6^{c}**	**33.8 ± 0.9^{d}**
n-6					
18:2n-6	14.3	15.6 ± 0.1^{a}	14.8 ± 0.2^{b}	13.8 ± 0.2^{c}	12.4 ± 0.2^{d}
18:3n-6	0.3	0.4 ± 0.0^{a}	0.8 ± 0.0^{b}	1.3 ± 0.0^{c}	1.8 ± 0.0^{d}
20:4n-6	0.8	0.5 ± 0.0^{a}	0.4 ± 0.1^{ab}	0.4 ± 0.0^{ab}	0.3 ± 0.0^{b}
n-3					
18:3n-3	4.2	7.5 ± 0.1^{a}	10.3 ± 0.3^{b}	12.9 ± 0.3^{c}	15.4 ± 0.4^{d}
18:4n-3	0.7	1.1 ± 0.0^{a}	2.9 ± 0.3^{b}	4.7 ± 0.0^{c}	6.7 ± 0.3^{d}
20:5n-3	1.5	1.1 ± 0.1^{a}	1.2 ± 0.1^{ab}	1.4 ± 0.0^{b}	1.4 ± 0.0^{b}
22:6n-3	10.5	5.8 ± 0.5	5.5 ± 0.1	5.2 ± 0.2	5.0 ± 0.2
Total PUFA	**34.6**	**34.3 ± 0.6^{a}**	**38.2 ± 0.7^{b}**	**42.0 ± 0.5^{c}**	**45.3 ± 0.7^{d}**
n-3:n-6 [1]	**1.1**	**0.9 ± 0.0^{a}**	**1.2 ± 0.0^{b}**	**1.6 ± 0.0^{c}**	**2.0 ± 0.0^{d}**

[1] n-3:n-6 is the ratio of ∑n-3 and ∑n-6 of n-3 and n-6 fatty acids, respectively, shown in this table. Values (mean ± SD, n = 3, consisting of fillets from five fish each) with different superscript letters within one row are significantly different ($p < 0.05$), values without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Materials and Methods. Initial data (n = 1, consisting of five livers) was not statistically analyzed.

Dietary fatty acid content linearly correlates with fatty acid content in fillet

Linear regression analysis of the fatty acid content (in mole%) in the diet (Table 3) and the fatty acid content (in mole%) in fillet (Table 3-7) is shown Fig. 3-2. In terms of ALA, SDA, and DHA the content of these fatty acids in the diet linearly correlates with the fatty acid content in the fillet with R^2-values in the range of 0.52-0.99. Fillet contents of ALA were always below the dietary content. Similar effects were found for SDA except for the diet with the lowest SDA content (FV). DHA had a higher content in fillet than in the diet. In contrast, EPA fillet content was negatively correlated with dietary EPA.

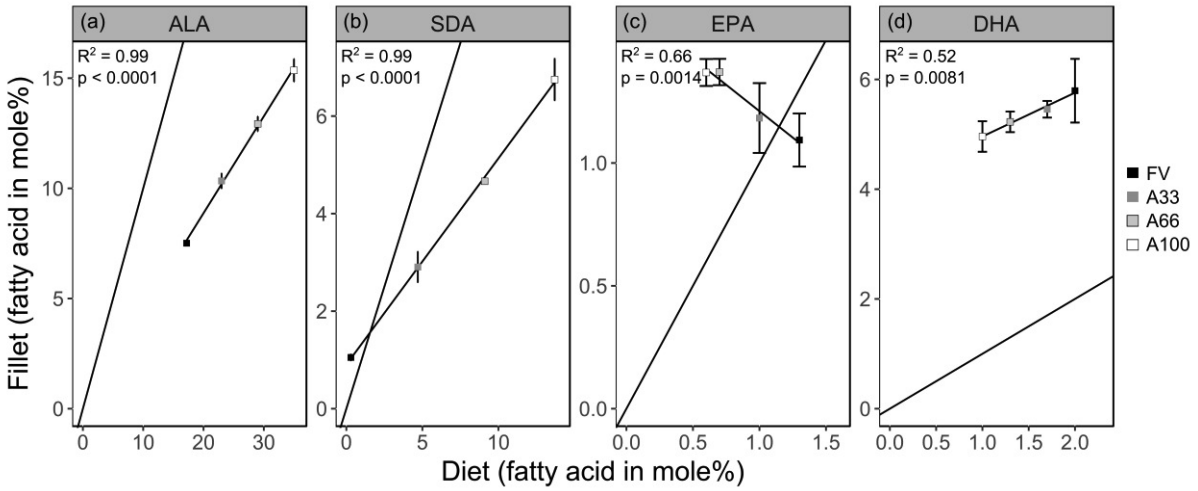

Fig. 3-2 Dietary fatty acid content in relation to the fillet fatty acid content. Figure shows values for (a) ALA, (b) SDA, (c) EPA and (d) DHA (in mole%) of rainbow trout fed the experimental diets FV, A33, A66 and A100. The additional line represents the line of equality. Error bars represent mean ± SD (n = 3).

Dietary Ahiflower oil affects fatty acid composition of liver

The fatty acid composition of the liver was affected by dietary Ahiflower oil (Table 3-8). Fish fed with the diet A100 showed a significantly ($p < 0.05$) lower hepatic monounsaturated fatty acid content compared to fish fed with the diets FV and A33. In addition, fish fed with the A66 diet had also significantly lower levels of these fatty acids than fish fed with the control diet FV. In contrast, the PUFA level was significantly ($p < 0.05$) increased in A66 and A100 compared to FV due to increased content of n-3 fatty acids (ALA, SDA, EPA and DHA). Consequently, the n-3:n-6 ratio significantly ($p < 0.05$) increased from FV to A100. A33 showed no consistent pattern as fatty acid values were mostly in between values of FV and A66 except for ALA, SDA, and EPA, which were significantly higher than FV. Saturated fatty acid levels were not altered by the dietary treatments.

Hepatic mRNA steady state levels

Fatty acyl desaturase 2a (delta-6) (*fads2a(d6*)), fatty acyl desaturase 2b (delta-5) (*fads2b(d5*)) and *cpt1a* remained on constant levels regardless of the dietary treatment (Fig. 3-3). *Cpt1c*, in contrast, was downregulated in A66 and A100 and thus, differed significantly ($p < 0.01$) from FV and from A33.

Table 3-8. Fatty acid composition (in mole%) of liver of rainbow trout fed the experimental diets FV, A33, A66 and A100 for 56 days.

Fatty acid	Initial	FV	A33	A66	A100
14:0	0.6	1.8 ± 0.2	1.6 ± 0.1	1.6 ± 0.1	1.5 ± 0.2
16:0	18.6	22.5 ± 0.3	22.0 ± 1.0	22.5 ± 0.9	22.4 ± 1.2
18:0	5.8	7.1 ± 0.4	7.3 ± 0.0	7.5 ± 0.2	7.6 ± 0.1
Total SFA	**26.0**	**32.0 ± 0.2**	**31.3 ± 1.0**	**31.8 ± 0.7**	**31.7 ± 1.2**
16:1	0.7	4.6 ± 0.4	4.4 ± 0.3	4.0 ± 0.6	3.6 ± 0.4
18:1	19.6	22.5 ± 0.6^{a}	19.8 ± 1.6^{ab}	17.8 ± 1.2^{b}	16.5 ± 1.1^{b}
Total MUFA	**23.7**	**31.8 ± 1.0^{a}**	**28.3 ± 1.9^{ab}**	**25.4 ± 1.4^{bc}**	**23.3 ± 1.3^{c}**
n-6					
18:2n-6	7.1	8.2 ± 0.3^{a}	6.9 ± 0.2^{b}	6.2 ± 0.4^{bc}	5.5 ± 0.3^{c}
18:3n-6	0.1	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}
20:4n-6	4.3	2.1 ± 0.2	2.0 ± 0.5	1.9 ± 0.0	1.7 ± 0.2
n-3					
18:3n-3	1.6	2.1 ± 0.1^{a}	2.5 ± 0.1^{b}	3.2 ± 0.4^{bc}	4.2 ± 0.4^{c}
18:4n-3	0.3	0.3 ± 0.0^{a}	0.7 ± 0.0^{b}	1.0 ± 0.0^{c}	1.3 ± 0.1^{d}
20:5n-3	3.2	1.6 ± 0.1^{a}	2.7 ± 0.3^{b}	3.4 ± 0.1^{c}	3.8 ± 0.3^{c}
22:6n-3	29.8	16.2 ± 0.4^{a}	19.1 ± 2.0^{ab}	19.8 ± 0.9^{b}	20.2 ± 1.1^{b}
Total PUFA	**50.0**	**35.8 ± 0.9^{a}**	**39.9 ± 2.3^{ab}**	**42.4 ± 1.5^{b}**	**44.5 ± 2.4^{b}**
n-3:n-6 [1]	**3.0**	**1.9 ± 0.1^{a}**	**2.8 ± 0.3^{b}**	**3.3 ± 0.2^{b}**	**3.9 ± 0.1^{c}**

[1] n-3:n-6 is the ratio of ∑n-3 and ∑n-6 of n-3 and n-6 fatty acids, respectively, shown in this table. Values (mean ± SD, n = 3, consisting of five livers each) with different superscript letters within one row are significantly different ($p < 0.05$), values without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Materials and Methods. Initial data (n = 1, consisting of five livers) was not statistically analyzed.

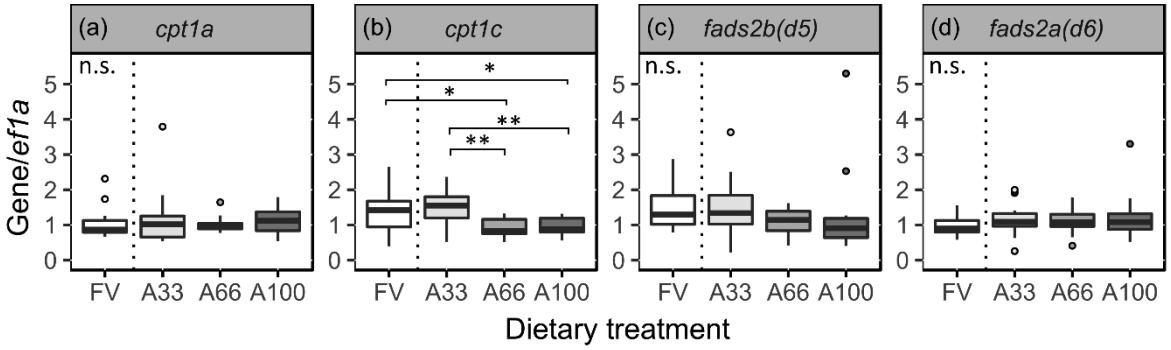

Fig. 3-3 Hepatic mRNA steady state levels. Rainbow trout were fed the experimental diets FV, A33, A66 and A100 for 56 days. Presented are boxplots of relative liver mRNA levels of (a) Carnitine palmitoyl transferase 1 a (*cpt1a*), (b) Carnitine palmitoyl transferase 1 c (*cpt1c*), (c) fatty acyl desaturase 2b delta-5 (*fads2b(d5)*) and (d) fatty acyl desaturase 2a delta-6 (*fads2a(d6)*). Liver mRNA gene expression was determined by qRT-PCR analysis and normalized to the housekeeping gene *ef1*α. Boxes represent values between the 25th and the 75th percentile; whiskers indicate 1.5 SD; medians are indicated by solid lines; outliers (above/below 1.5 SD) are indicated by solid circles. At the end of the experiment, 15 individuals per treatment were sampled in total (n = 15). Statistically significant differences between dietary treatments are represented by asterisks; $p < 0.05$ (*) < 0.01 (**) based on the statistical models described in Materials and Methods.

Discussion

Fish of all dietary groups performed well and more than doubled their body weight over the 56-day experimental period. Notably, fish fed with the Ahiflower oil diets had higher final body weights than fish fed with the FV diet. SGR and FCR in the present study were similar to those reported by Turchini and Francis [39] feeding fish oil- and linseed oil-based diets to rainbow trout. Cleveland et al. [40] substituted an SDA rich oil (Echium oil) for fish oil in rainbow trout with an initial body weight of about 32 g. Fish fed with Echium oil showed similar growth as fish fed with the fish oil diet. Furthermore, dietary Echium oil also had no effect on growth in Atlantic salmon parr [25], Atlantic cod [41], and arctic charr [42]. However, fish fed with the diet A100 showed significantly higher final body weights than fish fed with the FV diet, although the dietary content of saturated and monounsaturated fatty acids as well as EPA and DHA were lowest in this diet. This is contrasting the fact that oils low in saturated and monounsaturated fatty acids, such as Ahiflower oil, are considered to be not that effective for energy supply than other oils [43]. A factor possibly contributing to the higher final body weights may be the high content of ALA and SDA in the A100 diet. Bell et al. [44] suggest that high dietary levels of ALA might have been readily oxidized in Atlantic salmon. This could have been also the case for ALA and SDA in the present study. Thus, rainbow trout in the present study could have oxidized some of the ALA and SDA to compensate the lower MUFA levels in the A100 diet. Furthermore, high dietary ALA and SDA levels also seem to compensate for low dietary EPA and DHA levels without impairing fish growth. Thus, both, ALA and SDA, potentially not only served as a substrate for the n-3 LC-PUFA synthesis but also as an energy source. However, the significant increase of final body weights of fish fed with the A100 diet is not reflected to the same extent in the FCR and SGR values. Maybe further investigations should monitor fish for a longer period to clarify these observations.

Fish fed with the diets A66 and A100 showed increased spleen weights, and therefore, higher SSI values than fish fed FV and A33. A factor possibly contributing to the increased SSI values of fish fed A66 and A100 could be the higher PUFA contents in these diets. An increased PUFA content in pellets can lead to a more rapid peroxidation of fatty acids [45]. In turn, oxidized fat in pelleted feed increased SSI and HSI values in rainbow trout [46]. In addition to this fact, the spleen is involved in the immune defense [47,48]. In humans, the consumption of Ahiflower oil has immune modulating characteristics that are known from dietary marine oils [31]. In this study, a significant increase of the anti-inflammatory cytokine interleukin-10 was found in whole blood samples of humans consumed Ahiflower oil. Interleukin-10 is secreted inter alia by CD4 + T cells [49]. This type of cells can be also found in the spleen of rainbow trout [50]. It remains unclear whether the immune-modulating characteristics of the Ahiflower oil or the increased PUFA levels in the A66 and A100 diets per se might have affected the constitution of the spleen. Thus, a histological analysis is warranted for further studies with Ahiflower oil.

Inclusion of Ahiflower oil did not alter the chemical nutrient composition of whole body in rainbow trout. Whole body protein levels are in line with the uniform values for protein efficiency ratio (PER) and protein productive value (PPV) which were also not affected by dietary treatment. This is in accordance with a study in rainbow trout, reporting that moisture, lipid, and protein levels in whole body of fish were not affected by the dietary oil source [51]. Thus, our results indicate that Ahiflower oil can substitute up to 100% of the added oils in rainbow trout diets without altering the body composition and positively affecting the growth of rainbow trout.

It is well documented that the dietary fatty acid composition correlates with the fatty acid composition in fish tissue. Feeding plant oils devoid of LC-PUFA strongly alter the fillet fatty acid composition and decrease EPA and DHA contents [14–17]. In the present study, only the main fatty acid groups - saturated, monounsaturated fatty acids and polyunsaturated fatty acids - of experimental diets were reflected in the fillets of our rainbow trout. However, the contents of individual fatty acid in tissue samples were different to their respective dietary contents. The decrease of the dietary EPA and DHA levels due to the Ahiflower oil inclusion was not reflected in the liver and the fillets of fish fed with the diets A66 and A100. In contrast, with Ahiflower oil, the levels of these two fatty acids increased in the liver, and so did those of EPA in the fillets. These findings suggest an increased endogenous biosynthesis in fish fed with the diets A66 and A100. Rainbow trout are able to biosynthesize n-3 LC-PUFA endogenously [18]. This biosynthesis can be regulated via a feedback mechanism by dietary LC-PUFA. A diet deficient in these fatty acids, can enhance the biosynthesis of LC-PUFA [52]. Therefore, it is possible that the low contents of dietary EPA and DHA in A66 and A100 enhanced the n-3 LC-PUFA biosynthesis in fish. In addition, these findings are in line with results observed by Miller et al. [25], feeding Echium oil to Atlantic salmon parr. In their study, DHA levels in white muscle tissue were similar to those of a fish oil diet, indicating an increased biosynthesis of the n-3 LC-PUFA due to high dietary SDA levels. Therefore, it is possible that the reputed rate-limiting first delta-6-desaturase step in the biosynthesis pathway [53] could have been bypassed by dietary SDA in A66 and A100, thereby increasing the synthesis of EPA. In contrast, Cleveland et al. [40] assume that the availability of substrate might be the limiting factor in the n-3 LC-PUFA biosynthesis. In their study with rainbow trout, n-3 LC-PUFA contents in fish tissue were comparable between feeding linseed oil (rich in ALA) and Echium oil (rich in SDA). However, dietary content of ALA was markedly higher in the linseed oil diet than in the Echium oil diet. In contrast, Ahiflower oil contains higher ALA and SDA levels than Echium oil [28]. This, in turn, indicates that in the present study SDA as well as the high amount of ALA due to Ahiflower oil inclusion might have increased liver and fillet EPA of fish fed A66 and A100. Whatever the underlying mechanisms, present data support the hypothesis that inclusion of Ahiflower oil enhances n-3 LC-PUFA synthesis.

A preferential deposition and retention of DHA seem to have occurred in fillet, regardless of the dietary content of this fatty acid. As a result, all fillet DHA levels were higher than the content in the respective diet. This phenomenon has been observed in previous studies feeding different oil sources to Atlantic salmon [15,54]. In comparison to other dietary n-3 fatty acids that were readily oxidized, DHA has been shown to be relatively resistant to β-oxidation [55]. Further, Bell et al. [15] suggest that selective deposition and retention of DHA might be associated with the specificity of the enzymes that incorporate DHA into lipids. Thus, under the conditions investigated, an interaction of preferential retention and/or biosynthesis of EPA and DHA from dietary SDA presumably occurred in rainbow trout fed Ahiflower oil.

Studies in Atlantic salmon [55] and rainbow trout [56] indicate that enhanced LC-PUFA synthesis in liver only marginally affects the final fillet fatty acid composition. This is only partly applicable to our study, because EPA in liver and fillet was significantly increased in A66 and A100 compared to FV, albeit to a lower extent in the fillet than in the liver. However, DHA contents of A66 and A100 were significantly higher in liver but lower in fillet compared to FV. A possible explanation might be the duration of converting ALA and SDA, respectively, to EPA and DHA via the endogenous biosynthesis pathway. Bell et al. [44] fed differing levels of rapeseed oil as a substitute for fish oil (0-100%) to Atlantic salmon. Subsequently, all fish received a fish oil finishing diet. After four weeks, all groups reached similar EPA contents in flesh. In contrast, it took twelve weeks for the formerly 100% rapeseed oil group to reach DHA contents of the 0% rapeseed oil group. This corresponds to the fact that DHA is synthesized very slowly from ALA in rainbow trout [18]. Therefore, it could be possible that the experimental period of 56 days in the present study was too short for A100 fish to recover the depletion of fillet DHA. Thus, it remains unclear whether fish of A66 and A100 could have recovered DHA via increased DHA biosynthesis in liver, if the experimental period had been longer.

Expression of genes encoding proteins involved in fatty acid biosynthesis can be affected by dietary fatty acids in both mammals [57,58] and rainbow trout [59]. Dietary PUFA are known to regulate the delta-5- and delta-6-desaturase via a feedback mechanism [52]. For example, high dietary LC-PUFA, namely EPA and DHA, can decrease delta-5- and delta-6-desaturase gene expression and thereby reducing the n-3 LC-PUFA biosynthesis and vice versa [60]. Similar effects were found for Echium oil having high amounts of C18-PUFA (e.g. SDA and 18:3n-6). According to recent literature, feeding Echium oil to Atlantic salmon [27] and *Lates calcarifer* [61] resulted in liver delta-6-desaturase expression higher than fish oil and lower than vegetable oil (canola oil/rapeseed oil). In the present study, all dietary treatments resulted in similar *fads2a(d6)* mRNA steady state levels. However, we used a blend of both, fish and vegetable oils as a control group (FV). Thus, the higher content of EPA and DHA in the FV diet possibly led to a reduction of the *fads2a(d6)* mRNA expression in these fish. Simultaneously,

the high contents of SDA and 18:3n-6 in the A100 diet might have impaired *fads2a(d6)* gene expression, although content of EPA and DHA were lowest in this diet.

As already mentioned, as part of the Sprecher pathway, the delta-5-desaturation of 20:4n-3 results in EPA [62]. Miller et al. [27] described that the increased delta-5-desaturase gene expression due to high dietary SDA increased hepatic EPA levels in seawater Atlantic salmon. In the present study, we also found the highest EPA levels in liver and fillet samples of fish fed diets with high dietary SDA content (A66 and A100). Simultaneously, A66 and A100 had the lowest dietary EPA levels. Thus, we hypothesized that an increase in dietary SDA enhances the biosynthesis of EPA in tissues of fish fed with the diets A66 and A100. Therefore, we expected that the hepatic delta-5 gene expression in fish fed with these diets was significantly increased. Contrasting our expectations, EPA tissue levels were not supported by the hepatic *fads2b(d5)* mRNA steady state levels as there were no significant differences among dietary treatments. An explanation could be the influence of EPA and DHA on the gene expression. By the time of final sampling, liver EPA and DHA levels were significantly higher in fish fed with the A66 and A100 diets compared to livers of fish fed with the diet FV. A negative feedback loop of LC-PUFA on the expression of genes centrally involved in the biosynthesis of these fatty acids has been reported in zebrafish [63]. In addition, Greene and Selivonchick [64] assume that rainbow trout might have a physiologically optimum level of EPA. As EPA fillet levels were highest in fish fed with A66 and A100 by the time of sampling, they may have reached a physiological optimum level of this fatty acid. This factor combined with the high levels of EPA and DHA in the livers of the A66 and A100 fed fish might have led to a downregulation of the *fads2b(d5)* mRNA expression, resulting in similar values among dietary treatments.

Carnitine palmitoyl transferase (CPT1) is centrally involved in the mitochondrial β-oxidation. Two of its isoforms are CPT1a and CPT1c. They are important for the uptake of long-chain fatty acids into mitochondria [65]. The A100 diet had the lowest monounsaturated fatty acid and highest ALA content of all diets. However, this was not reflected to the same extend in terms of the fillet fatty acid composition of fish fed with A100. Previous studies indicated that when certain fatty acids are provided at high dietary amounts, for example ALA in A66 and A100, they can be readily metabolized and catabolized by β-oxidation [44]. Thus, we expected that increasing levels of Ahiflower oil result in increased β-oxidation of ALA. However, mRNA levels of *cpt1a* remained largely unaffected by the dietary treatments but *cpt1c* was significantly downregulated in A66 and A100. Therefore, it remains unclear why *cpt1a/c*, as an indicator for β-oxidation, was either unaffected or decreased in response to the dietary treatment, respectively.

Dietary inclusion of Ahiflower oil increases the content of ALA and SDA in fillet of rainbow trout. Simultaneously, the content of dietary 18:2n-6 decreases in a stepwise-manner (FV > A33 >

A66 > A100). This results in an increased n-3:n-6 ratio in fillet of rainbow trout, which could be also relevant in terms of human nutrition. Consumption of food rich in n-3 PUFA may decrease the risk of certain chronic diseases in humans [66,67] and it might have a protective effect on some forms of cancer, for example breast and prostate cancer [68,69]. Thus, present data support the hypothesis that the inclusion of dietary Ahiflower oil may improve the product quality of rainbow trout fillet for human consumption.

In conclusion, data of the present study suggest that Ahiflower oil can be included up to 100% into rainbow trout diets. Further, it does not alter body composition but increases growth of rainbow trout. It seems that the inclusion of Ahiflower oil enhances n-3 LC-PUFA synthesis. Furthermore, the content of DHA in fillet of fish fed with Ahiflower oil was similar to fish fed with FV possibly due to a preferential retention and/or biosynthesis of this fatty acid. Increasing dietary SDA did not alter gene expression of *fads2b(d5)*, *fads2a(d6)* and *cpt1a*. However, *cpt1c* was downregulated in A66 and A100. The utilization of Ahiflower oil increased the n-3:n-6 ratio in fillet dose-dependently toward a more favorable ratio for human nutrition. Overall, Ahiflower oil seems a promising candidate to substitute fish oil (and other vegetable oils) in rainbow trout diets. Future studies may use smaller trout and expand the duration of the experimental period to investigate if and to what extend DHA tissue levels are modulated by Ahiflower oil and whether there are any long-term effects on fish growth. Furthermore, the influence of Ahiflower oil inclusion into rainbow trout diets on sensory and shelf life needs to be investigated in future studies.

Acknowledgements

This work was funded by the Federal Office for Agriculture and Food (grant number 2815NA044). We would like to thank Dr. Hubert Wehage at the LUFA-itl in Kiel, Germany, for the fatty acid analysis and Jonas Schäler for his statistical advice. Further, we would like to express our gratitude to Adrian Schweiger, Carsten Rohwedder, Sandra Nevermann and Petra Rettmann for their assistance during the experimental period and in the lab. In addition, we are grateful for the guidance given by Michael Schlachter and all GMA colleagues for their help during sampling.

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CHAPTER 4

Combination of dietary Ahiflower oil and equol enhances LC-PUFA levels in rainbow trout tissues

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Modified version of the manuscript published in *Lipids*, 2018, 53 (11-12), 1069-1083, https://doi.org/10.1002/lipd.12117.

Abstract

Equol and Ahiflower oil have been shown to increase either eicosapentaenoic acid (20:5n-3, EPA) or docosahexaenoic acid (22:6n-3, DHA) levels in tissues of rainbow trout when applied individually. Thus, we investigated whether the combination of an Ahiflower oil-based diet and equol might increase both, EPA and DHA levels, in rainbow trout. Rainbow trout (87.1 ± 0.3 g) were fed five diets for eight weeks. A diet based on a blend of fish and vegetable oils (FV) served as a reference diet. The four experimental diets contained a blend of Ahiflower oil and vegetable oils (AV). The AV-diets were supplemented with equol by 0.0, 0.1, 0.2 and 0.3% DM of the diet (AV-C, AV-EQ1, AV-EQ2, AV-EQ3). The dietary treatments did not affect growth performance of fish and chemical nutrient composition of whole body samples. Fish fed with the equol diets showed dose-dependently increased liver weights and 18:0 liver levels. The content of EPA showed no consistent pattern between tissues but all AV-groups were characterized by higher liver EPA values than FV. DHA values of AV-EQ2 and AV-EQ3 were similar to FV in fillet, tended to be highest in whole body and were significantly higher in liver compared to FV. In contrast, mRNA steady state levels of fatty acyl desaturase 2a (delta-6) (*fads2a(d6)*) were not affected by the dietary treatments. In conclusion, the combination of dietary Ahiflower oil and equol (0.2 and 0.3%) seem to affect the fatty acid metabolism of rainbow trout positively in order to increase DHA tissue levels.

Keywords: Rainbow trout, Ahiflower oil, Equol, Oil Replacement, Fatty acid metabolism, long-chain polyunsaturated fatty acids (LC-PUFA)

Introduction

More than 50% of fish and aquatic plants for human consumption worldwide is supplied by aquaculture [1]. One of the most cultured salmonids is rainbow trout (*Oncorhynchus mykiss*) [1] and farming this carnivorous fish species was responsible for a large use of fish oil [2,3]. During the last decades, fish oil production remained on a stable level [4] while aquaculture production increased rapidly [5]. As a result, plant oils were considered the primary alternative to fish oil, leading to a gradual substitution of this ingredient in fish feed [2,6]. This shift in feed formulations had several advantages since plant oils are economically reasonable, easily available and devoid of harmful levels of dioxins and PCBs [7]. Further, many studies showed that dietary plant oils had little or no negative effect on growth and performance parameters in rainbow trout [8,9]. However, most plant oils exclusively contain α-linolenic acid (18:3n-3, ALA) as an omega-3 fatty acid (n-3) and are deficient in n-3 long-chain polyunsaturated fatty acids (LC-PUFA) [10]. Salmonids are able to synthesize eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) out of the precursor fatty acid ALA endogenously [11]. However, the utilization of dietary plant oils results in an adverse modification of the final content of these health-promoting n-3 LC-PUFA in fish [9,12,13]. A study on farmed Atlantic salmon showed that due to this shift, humans nowadays need to consume double the amount of salmon to meet the recommended EPA and DHA intake levels [14]. There are currently no plant oils available that can be considered as equivalent alternative to fish oil in terms of the fatty acid composition [15].

Ahiflower oil is derived from the plant *Buglossoides arvenis* and characterized by high amounts of stearidonic acid (SDA, 18:4n-3) [16]. Dietary SDA has been shown to be more readily bionconverted to n-3 LC-PUFA than ALA in humans and rainbow trout [17–19]. In our previous study, we showed that dietary Ahiflower oil significantly increased the content of EPA in livers and fillets of rainbow trout in comparison to fish fed a diet based on a fish and vegetable oil blend [20]. This effect was putatively due to bypassing the first step in the biosynthesis of LC-PUFA. However, DHA fillet levels were not increased in these treatments.

Equol is a metabolite of the phytoestrogen daidzein and synthesized by the intestinal bacterial metabolism in animals and humans [21,22]. Due to its similar chemical structure to estradiol it was shown to exert estrogenic effects in rainbow trout [23]. This could be an important characteristic, since genes involved in the biosynthesis of LC-PUFA such as the delta-6 desaturase and peroxisome proliferator-activated receptor alpha (PPARα) seem to be responsive to and activated by estrogen, respectively [24–26]. In our previous study, equol significantly increased DHA levels in whole body homogenates of rainbow trout [27]. However, no positive effect of equol on EPA tissue levels was found in this study.

Thus, in presented study we investigated, whether the combination of an Ahiflower oil-based diet and equol supplementation might increase both, EPA and DHA levels in rainbow trout and involved hepatic fatty acyl desaturase 2a delta-6 (*fads2a(d6)*) mRNA steady state levels.

Materials and Methods

Animals and experimental setup

The feeding trial was conducted at the facilities of the Gesellschaft für Marine Aquakultur mbH (GMA, Büsum, Germany) with monosex female rainbow trout obtained from a German fish farm (Forellenzucht Trostadt GbR, Germany). Fish were randomly distributed among the tanks of the recirculating aquaculture system (RAS, 20 m^3, turnover rate 2.4 h^{-1}, technical oxygen supply via oxygen cone) to acclimatize to the experimental conditions for two weeks. During this period, rainbow trout were fed on a commercial fish feed (AllerGold, 3 mm, AllerAqua) and a light/dark (14 h/10 h) cycle was adapted. Water purification system of the RAS contained drum filter (mesh size 20 µm), biofilter, ultraviolet (UV) disinfection and protein skimmer with ozone. At the start of the experiment, a total of 300 rainbow trout juveniles (87.1 ± 0.3 g initial body weight) were weighed individually and randomly distributed among 15 tanks of the RAS system. Each tank with a volume of 150 L was equally stocked with 20 fish (total initial biomass 1741.7 ± 6.5 g). The dietary treatments were randomly distributed in triplicates and the diets were hand-fed once per day for eight weeks. Each tank was bulk-weighed every 14 days to adjust the daily feed intake (DFI) level at 1.6% of biomass. The water parameters were in range for rainbow trout: temperature (14.8 ± 0.5 °C), oxygen (12.0 ± 0.2 mg L^{-1} O_2) and pH (7.5) were monitored continuously via probes. NH_4^+ (0.3 ± 0.2 mg L^{-1}), NO_2^- (0.7 ± 0.3 mg L^{-1}) (Microquant test kit for NH_4^+ and NO_2^-; Merck KGaA, Darmstadt, Germany) and salinity (4.2 ± 0.7 ‰) were measured daily. The experiment was carried out according to the national regulations for animal welfare (TierSchVersV) and the EU Directive 2010/63/EU for animal experiments. Furthermore, it was approved by the Ministry of Energy, Agriculture, the Environment, Nature and Digitalization (MELUND, Kiel, Germany; approved on 18 October 2017, project number V241-37421/2017).

Experimental diets

Fish were fed five diets with the same ingredient composition, differing only in their oil sources and the supplementation of equol. Thus, all diets were isonitrogenous and isoenergetic (Table 4-1). A fish and vegetable oil based diet served as a reference diet (FV). The four experimental diets contained a blend of Ahiflower oil and vegetable oils (AV). Three AV-diets were supplemented with equol by 0.1, 0.2 and 0.3% DM of the diet (AV-EQ1, AV-EQ2, AV-EQ3) in exchange with the respective amount of the filler ingredient bentonite. AV without supplementation served as negative control diet (AV-C).

Table 4-1. Ingredients and nutrient composition (in % of dry matter (DM)) of the experimental diets FV, AV-C, AV-EQ1, AV-EQ2 and AV-EQ3.

Diet	FV	AV-C	AV-EQ1	AV-EQ2	AV-EQ3
Ingredients [% DM]					
Fish meal [a]	15.0	15.0	15.0	15.0	15.0
Blood meal [b]	5.0	5.0	5.0	5.0	5.0
Feather meal [c]	5.5	5.5	5.5	5.5	5.5
Pea protein isolate [d]	12.0	12.0	12.0	12.0	12.0
Soy protein concentrate [e]	5.0	5.0	5.0	5.0	5.0
Wheat gluten [f]	14.0	14.0	14.0	14.0	14.0
Wheat starch [f]	22.0	22.0	22.0	22.0	22.0
Oil sources	***11.5***	***11.5***	***11.5***	***11.5***	***11.5***
Fish oil [g]	5.0	-	-	-	-
Rapeseed oil [h]	0.2	0.8	0.8	0.8	0.8
Linseed oil [i]	4.0	-	-	-	-
Palm fat [j]	0.5	4.4	4.4	4.4	4.4
Sunflower oil [h]	1.8	2.5	2.5	2.5	2.5
Ahiflower oil [k]	-	3.8	3.8	3.8	3.8
Gelatin [l]	1.5	1.5	1.5	1.5	1.5
Vitamin Mineral premix [m]	1.0	1.0	1.0	1.0	1.0
Calcium hydrogen phosphate [n]	0.5	0.5	0.5	0.5	0.5
α-Cellulose [o]	2.8	2.8	2.8	2.8	2.8
Lysine [p]	0.7	0.7	0.7	0.7	0.7
Methionine [q]	0.1	0.1	0.1	0.1	0.1
Bentonite [r]	3.4	3.4	3.3	3.2	3.1
Equol [s]	**-**	**-**	**0.1**	**0.2**	**0.3**
Nutrient composition [% DM]					
Dry matter [in % of diet]	86.3	87.7	87.3	87.3	87.2
Crude protein	52.0	51.5	51.0	51.0	51.8
Crude lipid	16.1	16.1	15.8	16.0	16.0
Crude ash	6.2	6.2	5.1	4.7	4.6
Total COH [in % DM] [t]	25.7	26.1	28.1	28.3	27.6
Gross energy [MJ/kg DM]	23.1	23.0	23.1	22.9	23.1

[a] Lean fish meal "low ash", Bioceval GmbH & Co. KG, Cuxhaven, Germany; [b] Daka porcine bloodmeal, Daka Denmark A/S, Løsning, Denmark; [c] GePro Goldmehl, GePro Geflügel-Protein Vertriebsgesellschaft mbH & Co. KG, Diepholz, Germany; [d] Emsland-Stärke GmbH, Emlichheim, Germany; [e] Euroduna Food Ingredients GmbH, Barmstedt, Germany; [f] KRÖNER STÄRKE GmbH, Ibbenbüren, Germany; [g] Bioceval GmbH & Co. KG, Cuxhaven, Germany; [h] Food store, Büsum, Germany; [i] Makana Produktion und Vertrieb GmbH, Offenbach a.d. Queich, Germany; [j] DF 100 PT-PV; EFG Elbe Fetthandel GmbH, Geesthacht, Germany; [k] Nature's Crops International, Kensington, Canada; [l] Gustav Ehlert GmbH & Co. KG, Verl, Germany; [m] Emsland-Aller Aqua GmbH, Golßen, Germany; [n] JRS Pharma GmbH & Co. KG, Rosenberg, Germany; [o] Mikro-Technik GmbH & Co. KG, Bürgstadt am Main, Germany; [p] Biolys, Evonik Industries AG, Essen, Germany; [q] MetAmino, Evonik Industries AG, Essen, Germany; [r] Castiglioni Pes y Cía, Buenos Aires, Argentina; [s] Equol: Cas No: 531-95-3, Xi'an Natural Field Bio-Technique Co., LTD, Xi'an Shaanxi, China; [t] Total COH = 100 – (crude protein + crude lipid + crude ash)

The fatty acid composition of the experimental diets is shown in Table 4-2. All diets were formulated to meet the dietary ALA requirement (0.7-1.0% of dry diet) of rainbow trout [10]. Further, in contrast to the FV diet, all AV-diets were below the recommended dietary EPA and DHA levels for this species (0.4-0.5% of dry diet, [10]). Dietary content of amino acids was calculated based on the amino acid contents of single ingredients in order to meet the amino

acid requirements of rainbow trout [10]. Pellets (4 mm diameter, 6 mm length) were produced with a pelleting machine (Type 14U175, Amandus Kahl, Hamburg).

Table 4-2. Fatty acid composition (in mole%) of the experimental diets FV, AV-C, AV-EQ1, AV-EQ2 and AV-EQ3.

	FV	AV-C	AV-EQ1	AV-EQ2	AV-EQ3
14:0	1.2	0.7	0.7	0.7	0.7
16:0	13.1	30.5	30.3	30.1	30.4
18:0	3.4	3.4	3.4	3.5	3.5
Total SFA	**18.5**	**35.3**	**35.1**	**35.0**	**35.3**
16:1	1.3	0.3	0.3	0.3	0.3
18:1	27.8	19.3	19.2	19.3	19.3
Total MUFA	**33.9**	**21.5**	**21.5**	**21.7**	**21.6**
n-6					
18:2n-6	24.9	23.1	23.1	23.1	23.1
18:3n-6	tr[a]	1.3	1.3	1.3	1.3
20:4n-6	0.2	tr	tr	tr	tr
n-3					
18:3n-3	17.2	12.4	12.7	12.4	12.4
18:4n-3	0.3	4.4	4.5	4.4	4.4
20:5n-3	1.3	0.5	0.5	0.6	0.5
22:6n-3	2.0	0.9	0.9	1.0	0.9
Total PUFA	**47.0**	**42.6**	**43.0**	**42.7**	**42.6**
ALA [% DM] [b]	**2.6**	**1.9**	**1.9**	**1.9**	**1.9**
EPA+DHA [% DM] [b]	**0.6**	**0.2**	**0.2**	**0.2**	**0.2**

[a] tr = traces (values < 0.1); [b] calculated from the FAME percentage data and the lipid content in the diets (in % DM), assuming 93% of total lipid to be fatty acids.

Sample collection

For the determination of growth performance parameters, all fish of a tank were bulk weighed at the beginning and the end of the 56 days-feeding period following 72 h of starvation. Feed conversion ratio (FCR), specific growth rate (SGR), protein efficiency ratio (PER) and protein retention efficiency (PRE) were calculated following the equations in Table 4-4. Acclimated residual fish were used to determine the initial status prior the start of the experiment. Physiological parameters are putatively sensitive and might be altered due to an extended starvation time. Thus, after the final bulk weighing, fish were re-fed their respective experimental diets for two additional days followed by the final sampling. The initial and final sampling were both performed following the same procedure: Prior to the sampling, fish were starved for 24 h, anesthetized and killed by a sharp blow on the head. Weight and length of all sampled fish was measured to calculate Fulton condition factor (K, Table 4-4). A pool sample of three fish per tank (initial: three fish) was used for the determination of the fatty acid and nutrient composition of whole body homogenates and stored at -20°C. Additionally, five fish (initial sampling: eight fish) per tank were sacrificed for pool samples of fillets and individual samples of spleen and liver. Pool samples of fillets were stored at -20°C. Spleen and liver weight was measured for calculation of spleen and hepatosomatic index (SSI and HSI).

Afterwards, a part of the liver was placed immediately into RNA later (Merck KGaA, Darmstadt, Germany) for the mRNA quantification of *fads2a(d6)* via quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). These samples were stored on ice during the sampling of each tank, followed by an overnight incubation at 4°C and finally stored at -20°C. The residual liver tissue of each tank was pool sampled and stored at -80°C. Prior to the analyses, whole body and fillet samples were freeze dried (alpha 1-4 LSC; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and homogenized with a knife mill (Grindomix, Retsch, Haan, Germany).

Chemical analysis

The proximate nutrient analysis and the determination of the energy content of diet, fillet and whole body homogenate samples were realized at the laboratory of the Gesellschaft für Marine Aquakultur mbH in Büsum, Germany. The proximate nutrient composition included dry matter (DM), crude ash, crude lipid and crude protein and was performed according to the EU guideline (EC) 152/2009 [28]. Samples were kept in a drying oven (ED53 9010-0078; Binder GmbH, Tuttlingen, Germany) at 103°C until constant weight to determine DM, following a four-hour incineration in a muffle furnace (LE 6/11/P300; Nabertherm, Lilienthal, Germany) at 560°C for determining the crude ash content. Crude lipid content was analyzed according to the Soxhlet method. Samples were hydrolyzed with hydrochloric acid (HYDROTHERM HT 6; C. Gerhardt GmbH & Co. KG, Königswinter, Germany) and lipids were extracted using petroleum ether (SOXTHERM® 416, Multistat/SX PC; C. Gerhardt GmbH & Co. KG, Königswinter, Germany). Crude protein (N x 6.25) was determined according to the methods of Kjeldahl including digestion, distillation (KjelDigester K-449, Scrubber K-415, KjelFlex 360; BÜCHI Labortechnik GmbH, Essen, Germany) and titration (877 Titrino plus; Deutsche METROHM GmbH & Co. KG, Filderstadt, Germany). Bomb calorimetry (C 200; IKA®-Werke GmbH & Co. KG, Staufen, Germany), calibrated with benzoic acid was used to measure the gross energy content.

Fatty acid composition

Fatty acid composition of oils, diets, liver, whole body homogenate and fillet samples was analyzed by LUFA-ITL GmbH in Kiel, Germany, using gas chromatography (GC) (Deutsche Gesellschaft für Fettwissenschaft e.V. (DGF), C-VI 10a). Fatty acid methyl ester (FAME) were prepared following the methods of Metcalfe and Schmitz [28], using saponification with methanolic NaOH and transmethylation of total lipids with boron trifluoride and methanol (DGF, C-VI 11a). The obtained FAME samples were separated by GC via split-injection (column: CP-Sil 88 50 m x 0.25 mm x 0.2 µm or similar) and detected using a flame ionization detector (FID) with helium as a carrier gas. The individual FAME were identified by comparison with certified

standard mixtures (18919-1AMP Supelco, F.A.M.E. Mix, Sigma-Aldrich) and FAME values were converted to moles. Fatty acid composition data is reported as mole% of total fatty acids.

RNA isolation and qRT-PCR

The RNAlater-stabilized liver samples (approximately 15 mg) were homogenized in a TissueLyser II (Qiagen, Hilden, Germany). Further, total RNA of these samples was isolated (Innuprep RNA Mini Kit, Analytik Jena, Jena, Germany) in accordance to the manufacturer's protocol. NanoDrop measurements (NanoDrop2000c; ThermoScientific, Waltham, MA, USA) were conducted in order to determine the RNA concentration and purity (ratios A260/280 and A260/230). Further, qRT-PCR measurements were carried out with the SensiFast SYBR No-ROX One-Step Kit (Bioline, London, UK) and a Rotor-Gene 6000 real-time PCR cycler (Corbett/Qiagen) to quantify the mRNA steady state levels of the selected target genes. A 1000 fold-diluted RNA sample was added by 18 µl to the Master Mix. The Master Mix consisted of 22.5 µl 2x SensiFAST-Mix, 2.48 µl DEPC treated water, 0.90 µl RNase inhibitor, 0.40 µl reverse transcriptase and 0.36 µl of each primer. 20 µl of this mixture was used for the analysis for each of the duplicates. The cycling conditions were as follows: at 49°C for 30 min, at 95°C for 10 min, 40 cycles of 95°C for 15 s, annealing temperature of the primer pair for 30 s and 72°C for 30 s, followed by a melt curve analysis from 60 to 99°C of 1°C increments. Sequences and the respective annealing temperatures of the primers are shown in Table 3. Relative mRNA concentrations were calculated using a standard curve. The mRNA steady state levels of the *fads2a(d6)* gene were normalized to the expression level of the housekeeping gene elongation factor 1 α (*ef1*α). This housekeeping gene was previously suggested as a gene reference in salmonid fish [30].

Table 4-3. Primer sequences (forward and reverse) and the respective annealing temperatures for mRNA measurements via qRT-PCR of samples from rainbow trout liver.

Gene	Primer	Sequence 5'→ 3'	Annealing Temperature (°C)
ef1α [a,*]	F	ACAAGCCCCTYCGTCTGCC	61
ef1α [a,*]	R	GCATCTCCACAGACTTSACCTCAG	61
fads2a(d6) [b,*]	F	GCTGGAGARGATGCCACGGA	61
fads2a(d6) [b,*]	R	TGCCAGCTCTCCAATCAGCA	61

[a] *ef1*α: Elongation factor 1 α; [b] *fads2a(d6)*: fatty acyl desaturase 2a (delta-6); * Geay et al. [67].

Statistical analysis

The data were evaluated using the statistical software R (2017), including the packages gdata, gplots, nlme, piecewiseSEM and multcomp. First of all, appropriate statistical models were defined: (1) statistical models based on general least squares [31] if values per tank were considered (final body weight (FBW), FCR, SGR, PER, PRE, DFI, nutrient and fatty acid

composition); (2) mixed models [32,33] with tank as random factor if values per fish (HSI, SSI, K, *fads2a(d6)*) were considered. The models included an artificial pseudo-influence factor (Treatment), representing a mixture of the actual factors diet, concentration and additive. This pseudo factor was necessary because the actual influence factors are not orthogonal (see [34]. The data were assumed to be normally distributed by a graphical residual analysis. Heteroscedasticity or homoscedasticity, respectively, was taken into account. A pseudo-R^2 was calculated, based on the model [35]. Further, in order to compare the dietary treatments, multiple contrast tests (e.g., see [36] were conducted.

Results

Growth performance and nutrient composition

All fish showed a 2.3 to 2-4-fold increase of their initial body weight within the eight weeks of the feeding trial. Furthermore, no significant differences among treatments for values of FBW, FCR, SGR, PER, PRE, SSI, K and DFI (Table 4-4) were found. However, liver weight of fish fed the diets AV-EQ2 and AV-EQ3 increased, resulting in significantly higher HSI values of these fish compared to HSI values of fish fed the other diets.

Table 4-4. Growth performance, feed intake, feed efficiency and biometric parameters of rainbow trout fed with the experimental diets FV, AV-C, AV-EQ1, AV-EQ2 and AV-EQ3 for eight weeks.

	FV	AV-C	AV-EQ1	AV-EQ2	AV-EQ3
IBW [1]	87.1 ± 0.2	86.8 ± 0.3	87.2 ± 0.3	87.2 ± 0.3	87.1 ± 0.3
FBW [2]	210.3 ± 4.0	209.9 ± 3.4	209.2 ± 1.5	203.7 ± 2.4	203.3 ± 4.8
FCR [3]	1.02 ± 0.03	1.01 ± 0.02	1.03 ± 0.01	1.06 ± 0.01	1.05 ± 0.02
SGR [4]	1.57 ± 0.03	1.58 ± 0.03	1.56 ± 0.01	1.52 ± 0.02	1.51 ± 0.04
PER [5]	2.20 ± 0.06	2.19 ± 0.04	2.18 ± 0.01	2.12 ± 0.02	2.10 ± 0.04
PRE [6]	39.1 ± 0.7	39.1 ± 1.9	39.2 ± 0.6	38.1 ± 0.9	36.9 ± 1.1
HSI [7]	1.56 ± 0.21^{a}	1.68 ± 0.19^{a}	1.63 ± 0.18^{a}	1.99 ± 0.29^{b}	2.09 ± 0.41^{b}
SSI [8]	0.12 ± 0.03	0.15 ± 0.05	0.16 ± 0.05	0.13 ± 0.04	0.14 ± 0.05
K [9]	1.38 ± 0.13	1.36 ± 0.11	1.37 ± 0.09	1.32 ± 0.09	1.35 ± 0.09

Values (mean ± SD, IBW, FBW, FCR, SGR, PER, PRE: n = 3; HSI, SSI: n = 3 (5 fish/tank); K: n = 3 (8 fish/tank)) with different superscript letters within one row are significantly different ($p < 0.05$), values without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Material and methods.

[1] Average initial body weight [g].

[2] Average final body weight [g].

[3] Feed conversion ratio = feed intake [g]/weight gain [g] [10].

[4] Specific growth rate [% d^{-1}] = [ln (final body weight) – ln (initial body weight)]/feeding day x 100 [68].

[5] Protein efficiency ratio = weight gain [g]/protein intake [g] [69].

[6] Protein retention efficiency = 100 x {[(final body protein x final body weight) – (initial body protein x initial body weight)]/protein intake} [10].

[7] Hepatosomatic index = 100 x (liver weight [g]/body weight [g]) [70].

[8] Spleen somatic index = 100 x (spleen weight [g]/body weight [g]) [71].

[9] Fulton condition factor = 100 x (final body weight/final body length^3) [68].

Furthermore, in contrast to whole body homogenates (Table 4-5), the nutrient composition of fillets (Table 4-6) was affected by the dietary treatments. Fillets of fish fed the diet AV-EQ1 showed significantly higher dry matter, crude protein and gross energy contents compared to fillets of fish fed the reference diet FV. Dry matter and crude protein contents of AV-EQ1 fed fish were also higher than in fillets of AV-EQ2 fed fish, whereas crude protein levels were similar to AV-EQ3 fed fish. Dry matter, crude protein and gross energy contents in fillets of fish fed the diets AV-C did not differ significantly.

Table 4-5. Nutrient composition of whole body homogenate (in % wet weight (WW); gross energy in MJ/kg WW) of rainbow trout before the experiment (Initial) and after being fed the experimental diets FV, AV-C, AV-EQ1, AV-EQ2 and AV-EQ3 for eight weeks.

	Initial	FV	AV-C	AV-EQ1	AV-EQ2	AV-EQ3
Moisture	72.9	69.2 ± 0.3	70.1 ± 0.7	69.6 ± 0.0	70.8 ± 1.2	69.9 ± 0.4
Crude ash	2.8	2.1 ± 0.0	2.2 ± 0.2	2.1 ± 0.1	2.3 ± 0.2	2.1 ± 0.1
Crude protein	16.4	17.2 ± 0.1	17.3 ± 0.4	17.3 ± 0.1	17.3 ± 0.3	17.1 ± 0.1
Crude lipid	7.9	11.4 ± 0.2	10.5 ± 0.6	11.0 ± 0.2	9.6 ± 1.1	10.8 ± 0.4
Gross energy [MJ/kg]	6.9	8.6 ± 0.1	8.4 ± 0.3	8.5 ± 0.1	8.0 ± 0.5	8.4 ± 0.1

Values (mean ± SD, n = 3) without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Material and methods. Initial data (n = 1, consisting of three fish) was not statistically analyzed.

Table 4-6. Nutrient composition of fillets (in % wet weight (WW); gross energy in MJ/kg WW) of rainbow trout before the experiment (Initial) and after being fed the experimental diets FV, AV-C, AV-EQ1, AV-EQ2 and AV-EQ3 for eight weeks.

	Initial	FV	AV-C	AV-EQ1	AV-EQ2	AV-EQ3
Moisture	77.6	74.2 ± 0.2^{a}	73.9 ± 0.4^{ab}	73.7 ± 0.1^{b}	74.2 ± 0.1^{a}	73.7 ± 0.2^{ab}
Crude ash	1.4	1.3 ± 0.0	1.3 ± 0.0	1.3 ± 0.0	1.3 ± 0.0	1.3 ± 0.0
Crude protein	18.4	19.8 ± 0.1^{a}	20.0 ± 0.1^{ab}	20.2 ± 0.2^{b}	19.9 ± 0.2^{ab}	20.4 ± 0.1^{b}
Crude lipid	2.6	4.6 ± 0.1	4.8 ± 0.4	4.7 ± 0.2	4.5 ± 0.1	4.4 ± 0.1
Gross energy [MJ/kg]	5.4	6.6 ± 0.1^{a}	6.7 ± 0.1^{ab}	6.7 ± 0.1^{b}	6.6 ± 0.0^{a}	6.7 ± 0.1^{ab}

Values (mean ± SD, n = 3; tank pool samples of fillets of five fish/tank) without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Material and methods. Initial data (n = 1, consisting of fillets of eight fish) was not statistically analyzed.

Liver fatty acid composition

Saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) of livers of rainbow trout were affected by the supplementation of dietary equol supplementation (Table 4-7). 14:0, 16:1, 18:1 and total MUFA levels decreased, whereas 18:0 increased significantly with increasing dietary concentrations of equol. For the 18:0 this effect was shown in a stepwise manner (FV=AV-C>AV-EQ1>AV-EQ2>AV-EQ3). A similar pattern can be found in the n-6 fatty acid levels: Linoleic acid was significantly lower in livers of fish fed the diets AV-EQ2 and AV-EQ3 compared to the control diets (FV and AV-C). In contrast, liver levels of arachidonic acid (ARA, 20:4n-6) almost doubled from FV to AV-EQ3 fed fish. All AV-fed fish had significantly lower

ALA, higher SDA ($p < 0.05$ for AV-C fed fish) and higher EPA ($p < 0.05$ for AV-C and AV-EQ1 fed fish) liver levels compared to liver levels of fish fed the reference diet FV. In case of ALA, the levels decreased almost in a stepwise manner with AV-EQ3 fed fish showing the lowest ALA content. In addition, fish fed with the diets AV-EQ2 and AV-EQ3 showed significantly higher DHA levels in their livers compared to levels of fish fed with the diets FV and AV-C. This increase further resulted in significantly higher total PUFA and sum of EPA+DHA levels in livers of these fish compared to livers of fish fed the FV- and AV-C-diets.

Table 4-7. Fatty acid composition (in mole%) of livers of rainbow trout before the experiment (Initial) and after being fed the experimental diets FV, AV-C, AV-EQ1, AV-EQ2 and AV-EQ3 for eight weeks.

	Initial	FV	AV-C	AV-EQ1	AV-EQ2	AV-EQ3
14:0	0.6	1.8 ± 0.2^{a}	1.8 ± 0.2^{ab}	1.4 ± 0.1^{bc}	1.2 ± 0.1^{c}	1.1 ± 0.1^{c}
16:0	18.6	22.5 ± 0.3	23.0 ± 0.6	22.7 ± 0.3	21.2 ± 0.7	21.0 ± 1.0
18:0	5.8	7.1 ± 0.4^{a}	7.0 ± 0.4^{a}	8.5 ± 0.0^{b}	10.2 ± 0.4^{c}	10.9 ± 0.3^{c}
Total SFA	**23.6**	**29.6 ± 0.1**	**29.7 ± 0.7**	**30.2 ± 0.3**	**30.2 ± 0.5**	**30.6 ± 1.0**
16:1	0.7	4.6 ± 0.4^{a}	4.7 ± 0.5^{a}	4.1 ± 0.3^{ab}	3.8 ± 0.3^{ab}	3.3 ± 0.2^{b}
18:1	19.6	22.5 ± 0.6^{a}	21.0 ± 1.1^{a}	17.6 ± 1.2^{b}	16.5 ± 0.7^{b}	16.2 ± 0.6^{b}
Total MUFA	**23.7**	**31.8 ± 1.0^{a}**	**29.8 ± 1.7^{a}**	**25.7 ± 1.3^{b}**	**24.9 ± 0.9^{b}**	**24.1 ± 0.6^{b}**
n-6						
18:2n-6	7.1	8.2 ± 0.3^{a}	8.0 ± 0.3^{a}	7.8 ± 0.6^{ab}	6.9 ± 0.2^{b}	6.8 ± 0.1^{b}
18:3n-6	0.1	0.2 ± 0.0^{a}	0.3 ± 0.0^{b}	0.3 ± 0.0^{ab}	0.3 ± 0.0^{ab}	0.2 ± 0.0^{ab}
20:4n-6	4.3	2.1 ± 0.2^{a}	2.6 ± 0.5^{ab}	3.1 ± 0.2^{abc}	3.4 ± 0.5^{bc}	3.9 ± 0.2^{c}
n-3						
18:3n-3	1.6	2.1 ± 0.1^{a}	1.4 ± 0.2^{bc}	1.4 ± 0.1^{b}	1.1 ± 0.1^{cd}	1.1 ± 0.1^{d}
18:4n-3	0.3	0.3 ± 0.0^{a}	0.5 ± 0.0^{b}	0.5 ± 0.0^{b}	0.4 ± 0.0^{ab}	0.4 ± 0.0^{ab}
20:5n-3	3.2	1.6 ± 0.1^{a}	2.0 ± 0.1^{b}	2.1 ± 0.2^{b}	2.0 ± 0.1^{ab}	2.0 ± 0.1^{ab}
22:6n-3	29.8	16.2 ± 0.4^{a}	16.7 ± 0.7^{a}	19.4 ± 1.6^{ab}	20.4 ± 1.0^{b}	20.7 ± 0.2^{b}
Total PUFA	**50.0**	**35.8 ± 0.9^{a}**	**37.6 ± 1.8^{ab}**	**41.1 ± 1.6^{bc}**	**41.8 ± 1.3$^{(b)c}$**	**42.2 ± 0.5^{c}**
EPA+DHA	**33.0**	**17.8 ± 0.4^{a}**	**18.7 ± 0.8^{ab}**	**21.5 ± 1.7^{bc}**	**22.4 ± 1.0^{c}**	**22.7 ± 0.2^{c}**

Values (mean ± SD, n = 3, consisting of livers from five fish each) with different superscript letters within one row are significantly different ($p < 0.05$), values without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Materials and methods. Initial data (n = 1, consisting of eight livers) was not statistically analyzed.

Whole body homogenate and fillet fatty acid composition

The fatty acid composition of whole body homogenates (Table 4-8) and fillets (Table 4-9) showed a similar pattern for most fatty acids. Total SFA and MUFA levels were higher and lower, respectively, for samples of fish fed the AV-diets. This was mainly due to an increase in 16:0 and a simultaneous decrease of 18:1 levels in the tissue samples of AV-fed fish. The 18:0 and 18:1 fillet levels were further increased and decreased, respectively in combination with high equol inclusion levels (AV-EQ2 and AV-EQ3). Furthermore, linoleic acid, 18:3n-6 and ALA were significantly increased and SDA significantly decreased in fillets and whole body homogenates of fish fed the AV-diets in comparison to samples of fish fed the diet FV. EPA levels in whole body homogenates were not affected by the dietary treatment. In contrast, EPA

levels in fillets of fish fed the AV-EQ3 diet were significantly lower than FV but significantly higher than fillet levels of fish fed the other AV-diets. Moreover, DHA and total PUFA levels in whole body homogenates were significantly affected by the supplementation of high dietary equol dosages. Fish fed the AV-EQ2 and AV-EQ3 diets showed significantly increased DHA and total PUFA levels compared to the FV fed fish. However, there was no consistent pattern in fillets of fish. The significantly lowest DHA level was found in the fillets of fish fed AV-EQ1. In comparison, DHA levels in fillets tended ($p = 0.063$) to be higher in AV-EQ2 and were significantly higher in AV-EQ3 fed fish, respectively compared to AV-EQ1 fed fish. DHA levels of fish fed the diet FV showed similar DHA levels as AV-EQ3 fed fish. Further, DHA fillet levels of fish fed the diet AV-C were in between levels of fish fed the diets AV-EQ1 and AV-EQ3. In contrast, significantly higher total PUFA levels were found for fish fed AV-EQ2 compared to fish fed the diet FV.

Table 4-8. Fatty acid composition (in mole%) of whole body homogenate of rainbow trout before the experiment (Initial) and after being fed the experimental diets FV, AV-C, AV-EQ1, AV-EQ2 and AV-EQ3 for eight weeks.

	Initial	**FV**	**AV-C**	**AV-EQ1**	**AV-EQ2**	**AV-EQ3**
14:0	1.7	1.8 ± 0.1^{a}	1.4 ± 0.1^{bc}	1.6 ± 0.0^{b}	1.4 ± 0.1^{bc}	1.3 ± 0.1^{c}
16:0	10.5	15.3 ± 0.1^{a}	17.2 ± 0.4^{bc}	17.7 ± 0.3^{b}	17.0 ± 0.2^{c}	17.5 ± 0.4^{bc}
18:0	2.7	3.9 ± 0.1	3.8 ± 0.1	3.8 ± 0.1	3.8 ± 0.1	4.0 ± 0.2
Total SFA	**15.6**	**21.7 ± 0.2**^{a}	**22.8 ± 0.6**^{ab}	**23.5 ± 0.3**^{b}	**22.7 ± 0.3**^{b}	**23.3 ± 0.5**^{b}
16:1	2.0	4.4 ± 0.2^{ab}	4.4 ± 0.2^{ab}	4.9 ± 0.2^{b}	3.6 ± 0.2^{a}	4.1 ± 0.4^{a}
18:1	39.6	36.9 ± 0.9^{a}	34.5 ± 1.0^{a}	33.6 ± 0.2^{b}	33.7 ± 0.9^{b}	33.3 ± 0.2^{b}
Total MUFA	**44.4**	**43.2 ± 0.8**^{a}	**43.6 ± 1.0**^{b}	**43.1 ± 0.3**^{b}	**42.3 ± 0.8**^{b}	**42.3 ± 0.6**^{b}
n-6						
18:2n-6	15.1	16.2 ± 0.1^{a}	16.8 ± 0.2^{b}	16.6 ± 0.1^{b}	17.4 ± 0.1^{c}	17.0 ± 0.4^{abc}
18:3n-6	0.4	0.4 ± 0.0^{a}	0.9 ± 0.0^{bc}	1.0 ± 0.0^{b}	0.9 ± 0.0^{bc}	0.8 ± 0.0^{c}
20:4n-6	0.5	0.3 ± 0.1^{a}	0.4 ± 0.1^{ab}	0.4 ± 0.0^{ab}	0.5 ± 0.0^{b}	0.4 ± 0.1^{ab}
n-3						
18:3n-3	4.3	7.3 ± 0.3^{a}	6.3 ± 0.1^{b}	6.3 ± 0.1^{b}	6.5 ± 0.3^{b}	6.3 ± 0.2^{b}
18:4n-3	0.9	1.0 ± 0.1^{a}	2.4 ± 0.1^{b}	2.3 ± 0.1^{bc}	2.1 ± 0.1^{bc}	2.0 ± 0.0^{c}
20:5n-3	1.1	0.7 ± 0.1	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	0.7 ± 0.0
22:6n-3	5.2	2.9 ± 0.5^{a}	3.3 ± 0.2^{ab}	3.2 ± 0.1^{ab}	3.8 ± 0.2^{b}	3.7 ± 0.1$^{(b)}$
Total PUFA	**29.6**	**30.8 ± 1.2**^{a}	**32.9 ± 0.5**$^{(b)}$	**32.7 ± 0.2**^{ab}	**34.2 ± 0.5**^{b}	**33.6 ± 0.6**^{b}
EPA+DHA	**6.4**	**3.6 ± 0.6**	**4.1 ± 0.2**	**4.0 ± 0.2**	**4.6 ± 0.3**	**4.4 ± 0.1**

Values (mean ± SD, n = 3, consisting of three fish each) with different superscript letters within one row are significantly different ($p < 0.05$), values without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Materials and methods. Initial data (n = 1, consisting of three fish) was not statistically analyzed.

Table 4-9. Fatty acid composition (in mole%) of fillet of rainbow trout before the experiment (Initial) and after being fed the experimental diets FV, AV-C, AV-EQ1, AV-EQ2 and AV-EQ3 for eight weeks.

	Initial	FV	AV-C	AV-EQ1	AV-EQ2	AV-EQ3
14:0	1.5	1.7 ± 0.1^{a}	1.4 ± 0.0^{b}	1.4 ± 0.0^{b}	1.4 ± 0.1^{b}	1.3 ± 0.0^{c}
16:0	11.3	15.8 ± 0.3^{a}	17.9 ± 0.4^{b}	18.2 ± 0.1^{b}	18.6 ± 0.3^{b}	18.6 ± 0.2^{b}
18:0	2.9	3.7 ± 0.1^{a}	3.8 ± 0.1^{a}	3.7 ± 0.1^{a}	4.0 ± 0.1^{b}	4.0 ± 0.0^{b}
Total SFA	**16.3**	**21.8 ± 0.4**^{a}	**23.6 ± 0.5**^{b}	**23.8 ± 0.0**^{b}	**24.4 ± 0.4**^{b}	**24.4 ± 0.2**^{b}
16:1	1.7	4.3 ± 0.1	4.3 ± 0.2	4.3 ± 0.1	4.1 ± 0.3	4.0 ± 0.2
18:1	39.6	33.6 ± 0.6^{a}	32.1 ± 0.7^{ab}	31.1 ± 0.6^{bc}	30.3 ± 0.2^{c}	30.5 ± 0.1^{c}
Total MUFA	**48.7**	**43.2 ± 0.8**^{a}	**40.4 ± 0.9**^{b}	**39.9 ± 0.8**^{b}	**38.9 ± 0.1**^{b}	**38.8 ± 0.2**^{b}
n-6						
18:2n-6	14.3	15.6 ± 0.1^{a}	16.5 ± 0.1^{b}	16.7 ± 0.3^{b}	16.6 ± 0.2^{b}	16.7 ± 0.2^{b}
18:3n-6	0.3	0.4 ± 0.0^{a}	0.9 ± 0.0^{b}	0.9 ± 0.1^{b}	0.8 ± 0.0^{b}	0.8 ± 0.0^{b}
20:4n-6	0.8	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.6 ± 0.0	0.6 ± 0.0
n-3						
18:3n-3	4.2	7.5 ± 0.1^{a}	6.3 ± 0.0^{b}	6.5 ± 0.2^{b}	6.4 ± 0.1^{b}	6.5 ± 0.2^{b}
18:4n-3	0.7	1.1 ± 0.0^{a}	2.4 ± 0.2^{b}	2.2 ± 0.2^{b}	2.2 ± 0.0^{b}	2.2 ± 0.0^{b}
20:5n-3	1.5	1.1 ± 0.1^{a}	0.6 ± 0.0^{b}	0.5 ± 0.1^{b}	0.6 ± 0.0^{b}	0.9 ± 0.0^{c}
22:6n-3	10.5	5.8 ± 0.5^{ab}	5.4 ± 0.2^{ab}	5.3 ± 0.0^{b}	5.7 ± 0.2$^{(a)}$	5.8 ± 0.1^{a}
Total PUFA	**34.6**	**34.3 ± 0.6**^{a}	**35.2 ± 0.4**^{ab}	**35.6 ± 0.8**^{ab}	**36.0 ± 0.4**^{b}	**36.0 ± 0.3**$^{(b)}$
EPA+DHA	**12.0**	**6.9 ± 0.6**^{abc}	**6.0 ± 0.2**^{bc}	**5.8 ± 0.1**^{c}	**6.3 ± 0.1**^{ab}	**6.7 ± 0.2**^{a}

Values (mean ± SD, n = 3, consisting of fillets from five fish each) with different superscript letters within one row are significantly different ($p < 0.05$), values without superscript letters within one row are not significantly different ($p \geq 0.05$) based on the statistical models described in Materials and methods. Initial data (n = 1, consisting of eight fillets) was not statistically analyzed.

Hepatic mRNA steady state levels

Hepatic *fads2a(d6)* mRNA steady state levels showed no significant differences among dietary treatments (Fig. 4-1).

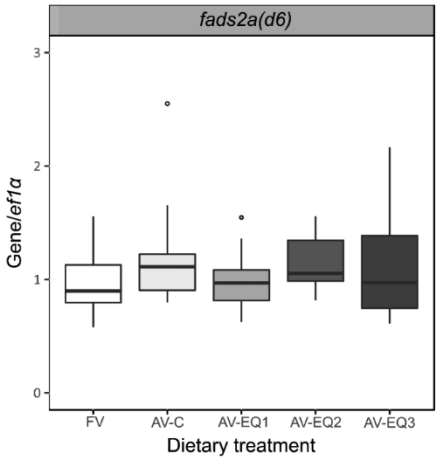

Fig. 4-1 Hepatic mRNA steady state levels. Rainbow trout were fed the experimental diets FV, AV-C, AV-EQ1, AV-EQ2 and AV-EQ3. Presented are boxplots of relative liver mRNA levels of fatty acyl desaturase 2a delta-6 (*fads2a(d6)*). Liver mRNA gene expression was determined by qRT-PCR analysis and normalized to the housekeeping gene ef1α. Boxes represent values between the 25th and the 75th percentile; whiskers indicate 1.5 SD; medians are indicated by solid lines; outliers (above/below 1.5 SD) are indicated by solid circles. At the end of the experiment, 15 individuals per treatment were sampled in total (n = 15). Statistical analyses was conducted based on the statistical models described in Materials and Methods.

Discussion

Effects of dietary treatments on growth performance and nutrient composition

All fish showed a 2.3 to 2-4-fold increase of their initial body weight within the eight weeks of the feeding trial without significant differences among treatments. Further, the growth parameters of fish in the present study can be considered as reasonable for rainbow trout at this size. FCR and SGR values in the present study were lower (FCR) and similar or higher (SGR) than values reported in experiments with rainbow trout of the same size [13,37]. The levels of EPA and DHA in the AV-diets in the present study were below the recommended dietary levels for rainbow trout [10]. However, rainbow trout are able to compensate low dietary EPA and DHA levels via the endogenous biosynthesis [11]. Thus, it seems that the fatty acids ALA and SDA of the AV-diets served as a substrate for the biosynthesis of EPA and DHA without impairing fish growth. This is in line with findings of our previous study using different inclusion levels of Ahiflower oil in rainbow trout diets [20]. Furthermore, the results of the present study also confirm our previous study that equol (0.1-0.3% of DM in the diet) combined with vegetable oil based-diets did not impair the growth of rainbow trout [27]. In contrast, other studies using bioactive substances such as genistein and resveratrol found reduced growth rates in fish [37,38]. This reduction was often related to a decrease in feed intake. A possible explanation could be the bitter taste of these secondary plant compounds as suggested by Torno et al. [39]. Equol is a metabolite of soybean originated isoflavone daidzein [40]. It has been described as more bitter than most of the isoflavones in soybeans [41]. In the present study, fish were fed on a fixed feeding ratio of 1.6% of biomass per day. Therefore, we can not fully exclude that equol might negatively affect feed intake and thus, growth, when included at higher dietary dosages and fed at higher daily feeding ratios.

PER and PRE values as well as the nutrient composition of whole body homogenates were not affected by the dietary treatment. This is in accordance to the results obtained in our previous study [27]. Thus, it seems that neither the dietary oil composition nor the supplementation of equol affected these performance parameters negatively in the present study. However, fillets of fish fed the diet AV-EQ1 showed significantly higher dry matter, crude protein and gross energy contents compared to fillets of fish fed the reference diet FV. The slight increase in dry matter and energy content is obviously due to the increase in crude protein. This increase in protein was only marginal and thus, did not alter the nutrient composition of whole body homogenates. Contrasting our findings, equol has been shown to reduce the protein synthesis rates in rainbow trout myocyte cells [42]. However, fillet protein levels were not decreased by dietary equol in the present study. Thus, further investigations about the effects on equol on protein metabolism are warranted.

Effects on LC-PUFA tissue levels

The replacement of the fish and vegetable oil blend (FV) with the blend of Ahiflower and vegetable oils (AV) altered the tissue fatty acid composition of rainbow trout in the current study. The AV-diets were characterized by lower EPA and DHA contents. However, DHA tissue levels were similar or even higher in AV-EQ2 and AV-EQ3 fed fish in comparison to levels of fish fed the diet FV. Further, EPA levels were increased in the livers of AV-fed fish. This is contrasting the findings of many studies, showing that the substitution of fish oil by vegetable oils decreases the EPA and DHA tissue levels in salmonids [43–45].

Specifically, the EPA and DHA liver levels were affected by the dietary treatments. The increased EPA levels in livers of fish fed the AV-diets are in accordance with results of our previous study using Ahiflower oil in rainbow trout diets [20]. We assumed that high amounts of dietary SDA could bypass the initial delta-6 desaturation step from ALA to SDA and thereby increase the levels of EPA. Thus, the high amount of SDA in the AV-diets seems to increase the efficiency of the biosynthesis of EPA. As there is no further increase of EPA when equol was added to the diet, it possibly does not have a direct effect on EPA tissue levels. In contrast, DHA levels were significantly increased in the AV-EQ2 and AV-EQ3 fed fish in comparison to livers of fish fed the control diets FV and AV-C. According to literature, the biosynthesis of DHA is increased in salmonids fed plant based diets [12,46,47]. However, fish do not achieve LC-PUFA levels of fish fed fish oil based diets [43]. As the liver DHA levels of AV-EQ2 and AV-EQ3 fed fish in the present study even exceeded those of fish fed the diets FV and AV-C, it seems very likely that the efficiency of the biosynthesis was markedly increased in these fish.

The biosynthesis of DHA requires *inter alia* a delta-6 desaturation from 24:5n-3 to 24:6n-3 [48]. The delta-6 desaturase has been shown to be responsive to estrogen and its expression was increased in the livers of ovariectomized rats treated with estradiol [25]. In the same study, this increased expression putatively increased DHA levels in plasma and liver of these animals. Equol exhibited an estrogenic potency in rainbow trout hepatocytes [23]. Thus, we hypothesized that equol would increase DHA levels in rainbow trout by increasing the expression of the delta-6 desaturase. In addition, phytoestrogens increased the expression of the gene *srebp1* in rainbow trout liver [49]. Schiller Vestergren et al. [50] assumed that *srebp* mediates the expression of genes involved in the biosynthesis of DHA, such as the delta-6 desaturase. Contrasting these assumptions, the mRNA steady state levels of *fads2a(d6)* were not affected by the dietary treatments. Fish were deprived of feed for 24 hours before the final sampling. Schiller Vestergren et al. [51] assume that the mRNA turnover might promptly respond to dietary alterations leading to constantly fluctuating mRNA levels. In addition, the highest hepatic DHA levels were found in fish fed the diets AV-EQ2 and AV-EQ3. Increased LC-PUFA levels decreased the expression of the delta-6 desaturase in zebra fish [52]. Thus, a negative feedback loop of DHA could have decreased the expression of *fads2a(d6)* in the

presented study. These factors might have affected the *fads2a(d6)* mRNA levels, leading to similar values among treatments.

However, there might be other mechanisms increasing DHA levels in fish fed the diets AV-EQ2 and AV-EQ3. PPARα is a transcription factor centrally involved in the peroxisomal β-oxidation [53], the synthesis step leading to DHA [54]. Kitson et al. [24] assume that estradiol might affect PPARα indirectly. Estrogen binds to the estrogen receptor alpha (ERα) and thereby may activate extracellular receptor kinase-mitogen activated protein kinase (ERK-MAPK). Thus, the phosphorylation and activity of PPARα is enhanced, resulting in an increased PPARα-dependent transcription of genes involved in the synthesis of DHA. Equol has also been shown to bind to ERα, although it has a higher affinity for the estrogen receptor beta (ERβ) [55]. Thus, the action of equol on DHA levels appears to be complex and various mechanisms seem to be possible. To understand the underlying mechanism, further research is needed on this topic.

However, the increased DHA levels in rainbow trout tissue due to the supplementation of equol seems to be dependent on the dietary fatty acid composition. In our previous study, we combined equol (0.1-0.3%) with plant oils and did not find an increase in hepatic DHA levels of rainbow trout [27]. Thus, the increased dietary SDA levels due to the Ahiflower oil inclusion might have increased the overall efficiency of the LC-PUFA biosynthesis, and combined with equol (0.2 and 0.3%), led to significantly higher DHA liver levels.

The increase in DHA liver levels corresponds well to the significantly decreased ALA levels in livers of fish fed AV-EQ3 compared to the AV-control group. Decreased ALA levels are putatively associated with an increased biosynthesis of LC-PUFA, since ALA is the precursor fatty acid and thus, serves as a substrate for the synthesis of these fatty acids [11]. Further, the MUFA levels in livers of fish fed the diet AV-EQ3 were significantly decreased. Both, MUFA and ALA can serve as a substrate for energy production in fish [11,13,56]. It could be possible, that ALA was preferably used for the endogenous biosynthesis of LC-PUFA and thus, higher amounts of MUFA were used for energy production.

The liver is the main tissue for the biosynthesis of EPA and DHA in fish [57]. However, DHA values of AV-EQ2 and AV-EQ3 fed fish were also increased in whole body homogenate samples in comparison to levels of fish fed the diet FV. In the fillet, DHA levels of fish fed the diets AV-EQ2 and AV-EQ3 were similar to levels of fish fed the diet FV. The difference in DHA levels between these two tissues might be explained by the fact that DHA is not only deposited in the liver and the fillet but also in brain, eyes and adipose tissue of rainbow trout [11]. Further, the synthesis of DHA in rainbow trout can be considered as rather slow [11]. Thus, by extending the experimental period, the DHA levels in fillets of fish might increase further and could possibly even exceed those of FV-fed fish. In contrast, the significantly increased EPA

liver levels in fish fed the AV-diets compared to fish fed the diet FV were not reflected in the whole body homogenate and fillet samples, indicating a higher retention of DHA in comparison to EPA. Low retention levels of EPA have also been found in studies with Atlantic salmon fed vegetable oil based diets [58,59]. A factor contributing to these results might be the different biological functions of these fatty acids in fish. DHA is preferably stored, as a major component, in the phospholipids in cell membranes [60]. In contrast, EPA is a precursor for the synthesis of both, DHA [48] and eicosanoids [61]. Eicosanoids of n-3 fatty acids, such as EPA, have anti-inflammatory characteristics. In contrast, n-6 fatty acids, such as ARA, promote the formation of eicosanoids with the opposite effects [reviewed in [61]]. Ishak et al. [52] suggest that EPA might inhibit the synthesis of ARA-based eicosanoids. Thus, the dose-dependent increase of ARA in the liver of fish fed the equol diets might have increased the formation of EPA-based eicosanoids. However, EPA fillet levels could be putatively further increased by including higher amounts of Ahiflower oil into the diets as shown by Fickler et al. [20].

General effects on the liver

Dietary equol at higher dosages (0.2 and 0.3%) exhibited putatively estrogen-like effects in the liver of rainbow trout in the presented study. The diets AV-EQ2 and AV-EQ3 increased the liver weight of fish, resulting in significantly higher HSI values in comparison to values of fish fed the other diets. These findings are similar to our previous study investigating the combination of dietary equol and plant oil-based diets in rainbow trout [27]. In addition, Siberian sturgeon fed a diet containing bioactive substances showed enlarged livers and hepatocytes [62]. Further, vitellogenin synthesis was increased in these fish. Vitellogenin synthesis has been found to be putatively mediated through ERβ [63]. A possible mechanism of action could be the binding of equol to estrogen receptors in the liver of fish. As already mentioned, equol preferably binds to ERβ [55]. Orally administered equol might be primarily transported to the liver via the entero-hepatic circulation, similar to estradiol. The liver is the tissue with the highest amounts of estrogen receptors in rainbow trout [64]. Thus, equol, having a structural similarity to the estrogen estradiol, might have exhibited an estrogenic potency and thereby increased vitellogenin synthesis in rainbow trout in the present study. Further, the 18:0 levels in livers of rainbow trout fed the equol diets increased dose-dependently. This effect has also been found in our previous studies with rainbow trout [27]. The enzyme elongase 6 mediates the elongation step from 16:0 to 18:0 [65]. This enzyme seems to be responsive to estrogen as it was increased in ovariectomized rats due to an estradiol application [66]. As 16:0 levels were decreased in AV-EQ2 and AV-EQ3 fed fish, the estrogenic potency of equol putatively enhanced the expression of elongase 6 and thereby increased the synthesis of 18:0 from 16:0 in fish fed the equol diets. However, further investigations are needed to examine the potential long-term effects of equol in rainbow trout.

Conclusion

In conclusion, the present study revealed promising results of combining Ahiflower oil and equol in rainbow trout diets to enhance the tissue LC-PUFA levels. Especially, the utilization of equol at 0.2 and 0.3% in combination with higher dietary SDA levels in the diet increased DHA contents in liver and whole body homogenates and thereby exceeded DHA tissue levels of fish fed the diet FV. Further, fish fed the diets AV-EQ2 and AV-EQ3 obtained similar DHA fillet levels as fillets of fish fed the diet FV. Thus, the combination of Ahiflower oil and dietary equol (0.2 and 0.3% of DM) might be a reasonable way to substitute dietary fish oil in rainbow trout diets.

Acknowledgements

We would like to thank Dr. Hubert Wehage at the LUFA-ITL in Kiel, Germany, for the fatty acid analysis. Further, the authors would like to thank Katharina Rosenhagen, Maximilian Horn, Anil Tellbüscher, Sandra Nevermann and Petra Rettmann for their assistance during the experimental period and in the lab. In addition, we are grateful for the guidance given by Michael Schlachter and all GMA colleagues for their help during sampling.

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GENERAL DISCUSSION

This thesis aimed to enhance the levels of eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) in rainbow trout (*Oncorhynchus mykiss*) which were fed vegetable oil-based diets in order to increase the nutritional value of these fish for human consumption. All approaches examined within this thesis (**chapters 1-4**) are based on the ability of rainbow trout to synthesize EPA and DHA endogenously.

1. Physiology

Physiologically essential highly unsaturated fatty acids like EPA and DHA have a variety of functions in fish. EPA and DHA are incorporated into phospholipids of the cell membrane in large amounts [1,2]. Specifically, DHA has a unique compact helical structure, giving this fatty acid a strong and flexible shape [1]. Apart from this, the characteristics of DHA are also important for tissues with visual and neural functions [1,3,4]. In contrast, EPA is a substrate for hormone-like compounds involved inter alia in the immune and inflammatory response (reviewed in [5,6]). Marine fish receive their high amounts of EPA and DHA via their natural diet: At the base of the food web, algae, mainly diatoms, produce these fatty acids, which are then transmitted via zooplankton to fish [6]. Freshwater fish, like rainbow trout, evolved in the ocean but later returned to the freshwater biosphere [6]. Thus, these fish depend on endogenously synthesizing long-chain polyunsaturated fatty acids (LC-PUFA) from their essential precursor fatty acids α-linolenic acid (18:3n-3, ALA) and linoleic acid (18:2n-6, LA). Due to this ability, rainbow trout rely mainly on dietary ALA and LA as substrates for the LC-PUFA synthesis. The NRC indicates a dietary ALA requirement level of 0.7-1.0% and LA of 1.0% of dry diet for rainbow trout, whereas for EPA and DHA only recommendations are given (0.4-0.5% of dry diet) [7]. Most of the diets investigated in this thesis contained EPA and DHA levels below the recommendations. However, there were no negative effects on growth and performance of fish which were fed diets containing vegetable oils in comparison to fish fed with the fish oil-based control diets (**chapters 3** and **4**). Rainbow trout which were fed the vegetable oil-based diets in **chapters 1** and **2** also showed reasonable growth rates for fish at this size. Thus, rainbow trout must have synthesized sufficient EPA and DHA de novo to meet their physiological essential fatty acid requirements and additionally enable optimum growth and health as described by Tocher [8].

The omega-3 (n-3) biosynthesis pathway described in the following is the most common [8] and has been confirmed for rainbow trout [9]. The studies within this thesis are based on this pathway. However, other options including delta-4 and delta-8 desaturations are possible. The delta-4 desaturation has been identified in rabbit fish and Senegalese sole [10,11], and the delta-8 desaturation in some marine fish species [12] as well as in rainbow trout and Atlantic salmon [13] (reviewed in [8]). However, all steps of the n-3 LC-PUFA biosynthesis pathway

take place in the endoplasmic reticulum with the final β-oxidation in the peroxisome. As recommended by Tocher [8], the common n-3 pathway should be divided into two separate pathways. The first part of the n-3 pathway comprises of the synthesis of EPA from ALA and requires two desaturation (delta-6 and delta-5) and one elongation step (Fig. GD-1). Desaturases insert a double bond in a particular position of the fatty acid [14], whereas elongases catalyze the extension of the fatty acid chain [15]. The second pathway, also known as the "Sprecher shunt" [16], includes the synthesis of DHA from EPA, involving an elongation of EPA to 24:5n-3 and a delta-6-desaturation followed by the final peroxisomal β-oxidation (chain shortening). The majority of the LC-PUFA biosynthesis takes place in the liver of rainbow trout and the same enzymes are operating in both pathways for the n-3 and n-6 fatty acids [17,18]. However, the competition between these two PUFA series has been shown to be only minimal in rainbow trout [19] as the enzymes involved prefer n-3 fatty acids over n-6 fatty acids [14].

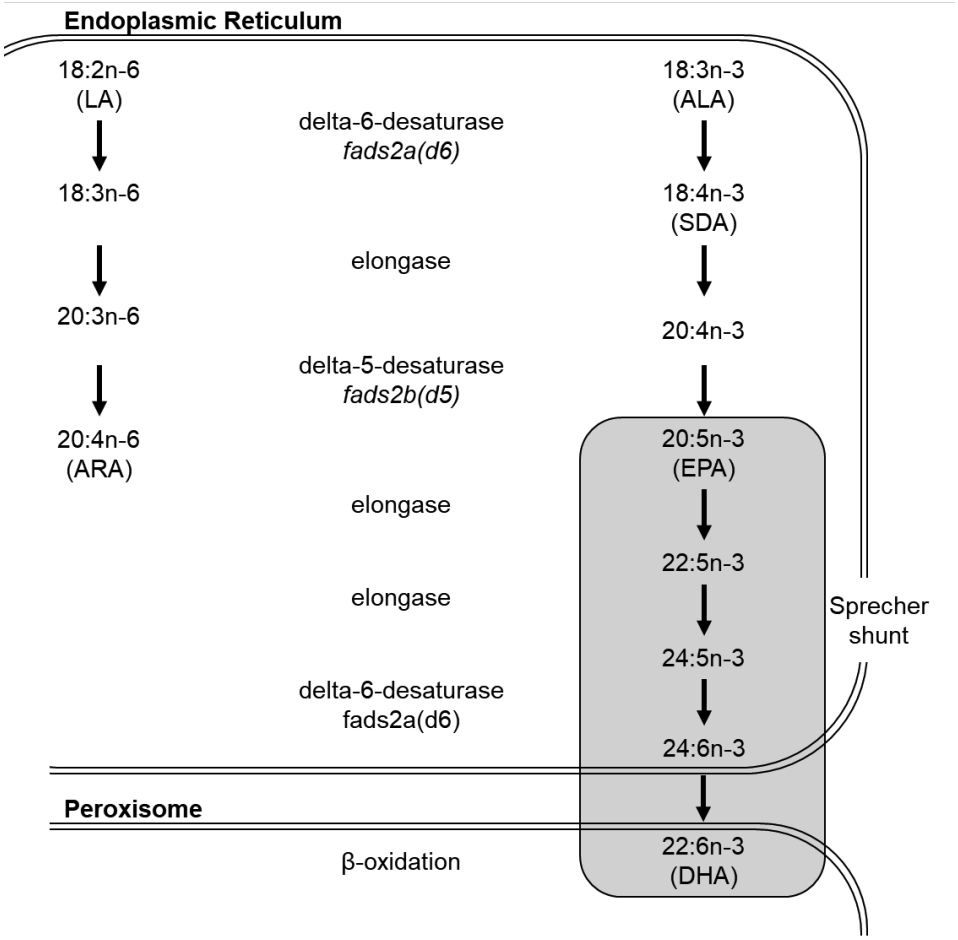

Figure GD-1 Pathways of n-3 and n-6 long-chain polyunsaturated fatty acids. Both pathways share the same desaturases and elongases. The n-6 fatty acid linoleic acid (LA) is converted via intermediates to arachidonic acid (ARA). The first part of the n-3 fatty acid biosynthesis involves the synthesis of EPA from ALA via SDA. The second part, the conversion of EPA to DHA via intermediates is also known as the Sprecher shunt. Except the peroxisomal β-oxidation, all steps take place in the endoplasmic reticulum. Adapted from Tocher et al. [8].

The promoter activities of these enzymes are regulated by transcription factors in the liver [14]. The transcription factors PPARα (peroxisome proliferator activated receptor alpha) and SREBP-1c (sterol regulatory element binding protein-1c) are centrally involved in the regulation of these enzymes [14]. The transcription factors and thus, the expression of genes involved in the biosynthesis of EPA and DHA can be regulated inter alia by nutrients (e.g. fatty acids) and hormones (e.g. estrogen) [14,20–23]. In the following, the relation between the final tissue fatty acid composition of rainbow trout and (1) the dietary fatty acid composition, (2) the utilization of bioactive substances and (3) the interaction of both (1) and (2), will be discussed. As the endogenous biosynthesis of LC-PUFA has evolved to satisfy the physiologically essential levels of these fatty acids in fish but does not necessarily lead to EPA and DHA levels high enough to meet the consumer demands [8], the approaches (1-3) will be evaluated regarding their potential to achieve an increased product quality of rainbow trout for human consumption.

1.1 Effect of dietary fatty acid composition on tissue fatty acid composition

It is well known that the tissue fatty acid composition resembles the dietary fatty acid composition in fish and that feeding vegetable oil-based diets low or devoid of EPA and DHA strongly influences the fillet fatty acid composition [24–26]. However, some fish can maintain tissue EPA and DHA contents at higher levels than derived via the diet. This is due to their ability to endogenously synthesize LC-PUFA. Substituting dietary fish oil with vegetable oils generally resulted in an increased biosynthesis of LC-PUFA in the liver [27–29]. Fish fed diets with gradually lower EPA and DHA levels in **chapter 3** showed increased levels of these fatty acids in the liver, indicating an increase in the LC-PUFA biosynthesis. In accordance, livers of fish fed with a diet based on Ahiflower and vegetable oils had increased EPA levels in comparison to fish fed with a diet based on fish oil and vegetable oils (in the following termed as “fish oil-based diet”) (**chapter 4**). In salmonids, the regulation of the LC-PUFA synthesis can be mediated by dietary fatty acids [30]. Dietary PUFA can regulate transcription factors such as PPARα and SREBP-1c (reviewed in [22]). The presence of these fatty acids leads to a negative feedback loop on the expression of genes involved in the biosynthesis [31,32]. In turn, the absence of LC-PUFA can relieve this suppression and thereby increase the expression of genes needed to synthesize EPA and DHA. Fish which are fed vegetable oil-based diets have been shown to increase the expression of genes involved in this biosynthesis pathway 2- to 3-fold (reviewed in [27,33]). For example, both the expression and the activity of the delta-6-desaturase were increased in salmonids fed vegetable oil-based diets in comparison to fish which were fed fish oil-based diets (reviewed in [34]). In this thesis, vegetable oil-based diets low in EPA and DHA did not lead to significantly increased hepatic mRNA steady state levels of the delta-6-desaturase (**chapters 1, 3** and **4**) and of the delta-5-

desaturase (**chapter 3**). However, the activity of the desaturases was not measured. In addition, it cannot be excluded that fatty acids might also affect the processes in the lipid metabolism via alterations in fluidity and microenvironments of the membrane as suggested by Tocher et al. [35].

Nevertheless, apart from the absence of dietary LC-PUFA, providing stearidonic acid (18:4n-3, SDA) instead of ALA could further enhance the efficiency to synthesize EPA, as the first delta-6-desaturation step of the biosynthesis can be bypassed. This hypothesis was investigated within this thesis by using a vegetable oil rich in SDA (Ahiflower oil). Figures GD-2 and GD-3 show EPA and DHA whole body homogenate levels of rainbow trout fed diets based on fish oil (FVO), vegetable oil (VO) and Ahiflower oil (AVO) (dietary fatty acid composition described in Table GD-1). A significant effect of SDA over ALA on EPA whole body homogenate levels of rainbow trout was only obtained with higher dietary EPA and DHA levels as well as higher growth rates due to a higher daily feeding ratio (2.0% and 1.6% of biomass per day, respectively) (Table GD-1). Thus, it seems that the beneficial effect of SDA might depend on the fatty acid composition of the diet and is possibly affected by the growth rates of fish. Varying dietary levels of EPA and DHA and different growth rates could have also led to contradictory findings about the beneficial effects of the SDA-rich Echium oil over common vegetable oils in salmonid diets [36–38].

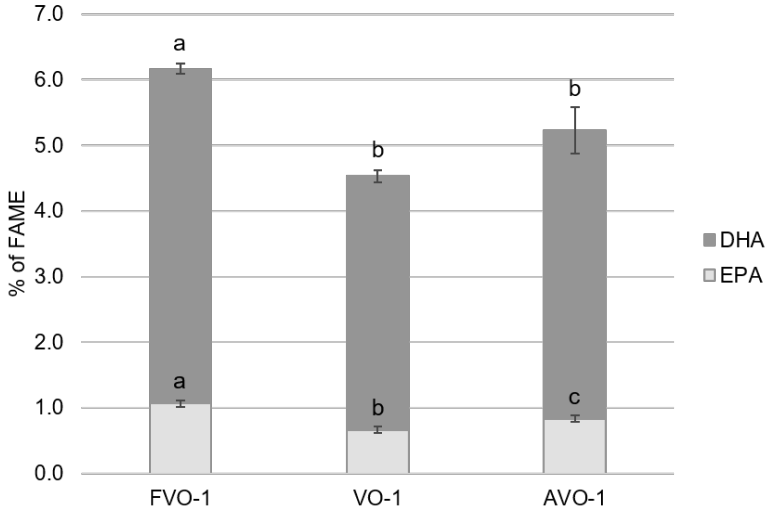

Fig. GD-2 EPA and DHA levels in whole body homogenates of rainbow trout fed the diets FVO-1 (based on: fish/vegetable oils), VO-1 (vegetable oils) and AVO-1 (Ahiflower/vegetable oils) for eight weeks. Values (mean ± SD, n = 3, consisting of three fish each) with different superscript letters are significantly different ($p < 0.05$), values without superscript letters are not significantly different ($p \geq 0.05$). Values were analyzed according to the statistical methods described in chapter 3 (p. 65).

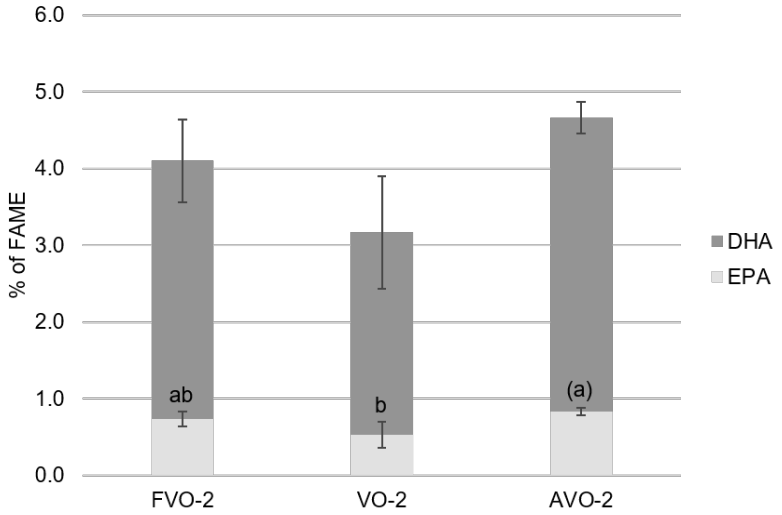

Fig. GD-3 EPA and DHA levels in whole body homogenates of rainbow trout fed the diets FVO-2 (based on: fish/vegetable oils), VO-2 (vegetable oils) and AVO-2 (Ahiflower/vegetable oils) for eight weeks. Values (mean ± SD, n = 3, consisting of three fish each) with different superscript letters are significantly different ($p < 0.05$), values without superscript letters are not significantly different ($p \geq 0.05$). Brackets indicate a tendency towards a statistical difference ($p < 0.1$). Values were analyzed according to the statistical methods described in chapter 3 (p. 65).

Table GD-1 Fatty acid composition (in % of FAME*) of the experimental diets FVO (based on fish/vegetable oils), VO (vegetable oils) and AVO (Ahiflower/vegetable oils) -1 and -2, respectively and the specific growth rate of rainbow trout fed these diets for eight weeks.

	FVO-1	VO-1	AVO-1	FVO-2	VO-2	AVO-2
ALA	14.7	15.0	11.3	17.1	16.7	12.5
SDA	0.7	0.2	3.9	0.3	0.1	4.4
ΣALA+SDA	**15.4**	**15.2**	**15.2**	**17.4**	**16.8**	**16.9**
ΣEPA+DHA	**5.9**	**2.1**	**2.1**	**3.7**	**1.6**	**1.6**
SGR[a]	2.23 ± 0.03	2.15 ± 0.01	2.12 ± 0.08	1.57 ± 0.03	1.56 ± 0.01	1.58 ± 0.03

* FAME = Fatty acid methyl ester; [a] Specific growth rate [% d^{-1}] = [ln (final body weight) – ln (initial body weight)]/feeding day x 100.

However, when dietary ALA and SDA levels are further increased, for example by using solely Ahiflower oil as a dietary oil source (**chapter 3**), there is a clear effect on the tissue LC-PUFA levels in rainbow trout. The interaction of decreased dietary EPA and DHA, and simultaneously increased ALA and SDA levels has improved the EPA liver and fillet levels of these fish. Thereby, these fish exceeded the EPA fillet levels (+25%) and reached similar DHA levels of fish fed with the fish oil-based control diet in **chapter 3**. These results are in contrast to the fact that the LC-PUFA synthesis generally does not compensate for the lack of dietary EPA and DHA leading to decreased levels of these fatty acids in fish which are fed vegetable oil-based diets in comparison to fish which are fed diets based on fish oil [27,39]. Briefly, the utilization of Ahiflower oil and thus, the modification of the dietary fatty acid composition, can increase the LC-PUFA levels of rainbow trout. However, this increase is limited and more innovative approaches are needed to further increase the efficiency of the LC-PUFA synthesis.

1.2 Effect of isoflavones on tissue fatty acid composition

An approach which can further stimulate the LC-PUFA biosynthesis is the utilization of isoflavones. Isoflavones have several possible mechanisms to affect the biosynthesis of EPA and DHA in rainbow trout. For example, isoflavones might increase the LC-PUFA tissue levels indirectly by protecting these fatty acids from oxidation. Genistein was found to develop antioxidant properties in endothelial cells: Genistein activated the transcription factor Nrf1 and thus increased the activity and expression of its target gene, the glutathione peroxidase which is involved in the cellular defense mechanisms of endothelial cells [40]. However, this might not be the only way to increase LC-PUFA tissue levels in rainbow trout. In addition to this, isoflavones can function, similar to PUFA, as ligands for PPARα [41,42]. PPARα can be activated by ligand binding, forming a heterodimer with the retinoid X receptor (RXR). This PPARα-RXR-complex binds to peroxisome proliferator response elements located within the promoters of the target genes and thereby activating their transcription [43]. In general, PPARα is involved in the regulation of the desaturases (delta-5 and delta-6) [14] as well as the peroxisomal β-oxidation (acyl CoA oxidase and D-bifunctional protein) [20,44]. All of these genes are necessary for the formation of DHA. This is in accordance with findings in **chapters 1** and **4** indicating that isoflavones mainly increase DHA and not EPA levels.

In addition to ligand binding, isoflavones can affect transcription factors and genes involved in the biosynthesis of LC-PUFA due to their estrogenic activity. Kitson et al. [20] describes that the endogenous hormone estrogen likely activates PPARα via two mechanisms: Estrogen could bind to the estrogen receptor alpha (ERα) and thus activates the extracellular receptor kinase-mitogen activated protein kinase (ERK-MAPK). Thus, PPARα activity is increased via ERK-MAPK-mediated phosphorylation. Another possible mechanism is the enhanced ligand allocation for PPARα. Estrogen could increase the intracellular concentration of ligands like PUFA and eicosanoids, thereby activating PPARα. Isoflavones have a similar chemical structure as the estrogen 17β-estradiol [45] (Fig. GD-4) and thus can bind to estrogen receptors [46,47]. In accordance, the isoflavones genistein, daidzein and biochanin A as well as the isoflavone metabolite equol showed an estrogenic potency in rainbow trout [48]. In addition to the above mentioned mechanisms of action, isoflavones might exert estrogenic effects in livers of salmonid fish also by inhibiting the metabolism of estrogen and thereby increasing the bioavailability of estrogen as suggested by Ng et al. [49]. Although, the estrogenic activity of the substances used in this thesis was not measured, it seems likely that the isoflavone metabolite equol among all investigated substances developed estrogen-like effects in rainbow trout, indicated by increased liver weights (**chapters 2** and **4**) and increased 18:0 fatty acid levels (**chapters 1, 2** and **4**).

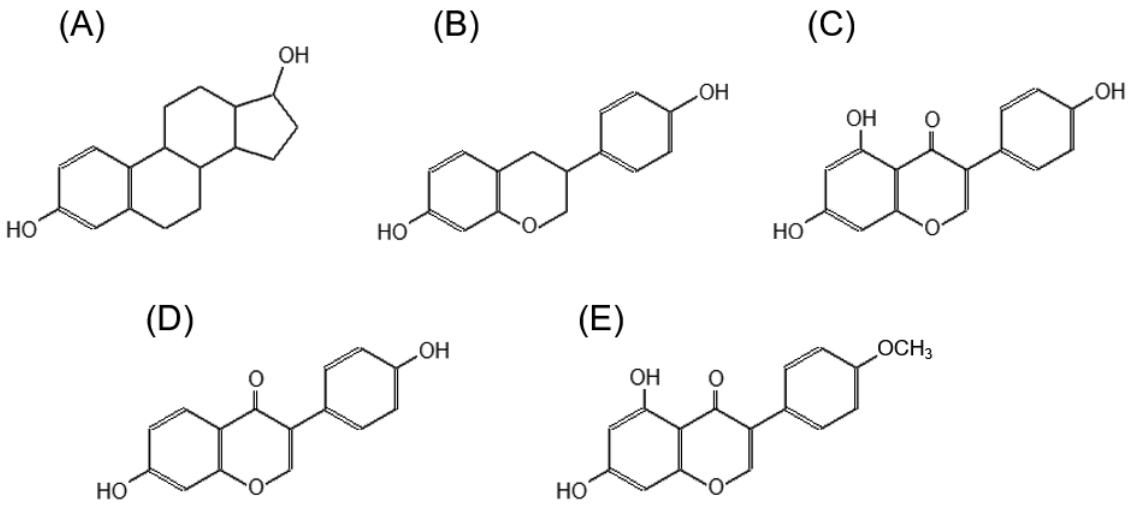

Fig. GD-4 Structure of 17β-estradiol (A), the isoflavone metabolite equol (B), and the isoflavones genistein (C), daidzein (D), and biochanin A (E). Adapted from Setchell et al. [50] and Medjakovic et al. [51].

Ronis et al. [52] fed soy protein isolate-based diets to rats and found increased expression of PPARα and PPARα-regulated genes such as CPT1 (carnitine palmitoyl transferase 1). In contrast to these findings, genistein and equol did not affect mRNA steady state levels of CPT1a and c (**chapter 1**). Moreover, *fads2a(d6)* mRNA steady state levels were either not affected (genistein: **chapter 1**, equol: **chapter 4**) or downregulated (equol: **chapter 1**) by these substances. In addition, protein levels of Fads2a(d6) were not altered by genistein and equol (**chapter 1**). Thus, it seems that equol exerts multiple effects in rainbow trout that cannot be explained by one single action.

In addition to PPARα, SREBP-1c is also an important transcription factor of genes involved in the biosynthesis of LC-PUFA [14]. For example, genistein inhibited the activation process of SREBP-1 and thus, downregulated genes regulated by SREBP-1 [53]. Li et al. [54] suggest that PPARα and SREBP-1c need to be activated simultaneously to increase the transcription of the gene encoding the delta-6-desaturase. Thus, it might be possible that isoflavones increase the activity of PPARα but simultaneously decrease the expression of genes mediated by SREBP-1c leading to a reduction of the PPARα-mediated effects on LC-PUFA levels. In accordance with these findings, the effects of genistein and equol on DHA were only marginal in whole body homogenates in **chapter 1** and not existent in tissues of rainbow trout in **chapter 2**. However, increased EPA and DHA liver levels due to equol application were found in **chapter 4**, indicating that the dietary fatty acid composition and thus, the dietary lipid source, might also play a role in the mode of action of equol.

1.3 Effect of different dietary fatty acid compositions in combination with isoflavones on the tissue fatty acid composition

Isoflavones and their metabolites are not solely able to function as a substitute for dietary fish oil. The utilization of these substances as an alternative to fish oil requires the inclusion of vegetable oils in the diets. However, it seems that the efficacy of isoflavones to increase DHA in rainbow trout depends on the dietary fatty acid composition. For example, different effects of the bioactive compound sesamin on LC-PUFA tissue levels due to varying dietary fatty acid compositions have been found in Atlantic salmon and barramundi (*Lates calcarifer*) [55,56]. The dietary levels of EPA and DHA as well as ALA and SDA might, therefore, play an important role. The diets used in **chapter 1** had higher EPA and DHA levels than those in **chapter 2**. In **chapter 1,** genistein and equol affected the tissue LC-PUFA levels, whereas in **chapter 2** no effects were found, although the dietary concentration of these substances was increased. Obviously, higher dietary levels of EPA and DHA increase the effect of isoflavones on LC-PUFA levels. This hypothesis seems to be confirmed as the combination of isoflavones (0.15%) with a diet based on fish and vegetable oils shows significantly increased DHA levels in the fillets (Fig. GD-5) and higher levels in the livers of rainbow trout (Fig. GD-6). In turn, it might be possible that diets low in EPA and DHA already fully exploit the endogenous LC-PUFA biosynthesis capacity and that isoflavones are not an adequate strategy to further increase LC-PUFA synthesis when combined with diets based on common vegetable oils.

The efficiency of the LC-PUFA biosynthesis pathway putatively has a “funnel-like” characteristic, as the relative activity of desaturases and elongases decrease with increasing chain length of the fatty acid [57]. Thus, by increasing the ratio of the conversion of ALA/SDA to EPA due to dietary Ahiflower oil, there might be more substrate available for the synthesis of DHA. This could explain the different findings concerning the effects of equol on the LC-PUFA biosynthesis in **chapters 2** and **4**, which are directly comparable as they were obtained within the same experiment. Equol (0.2 and 0.3%) combined with a diet based on Ahiflower oil significantly increased DHA levels in the livers of rainbow trout compared to rainbow trout fed the Ahiflower oil-based control diet in **chapter 4**. Contrasting these findings, equol combined with diets based on common vegetable oils did not affect the LC-PUFA tissue levels of rainbow trout. All diets had similar levels of ΣALA+SDA, but Ahiflower oil-based diets had higher levels of SDA and lower levels of ALA (**chapter 4**) compared to the diets based on common vegetable oils (**chapter 2**).

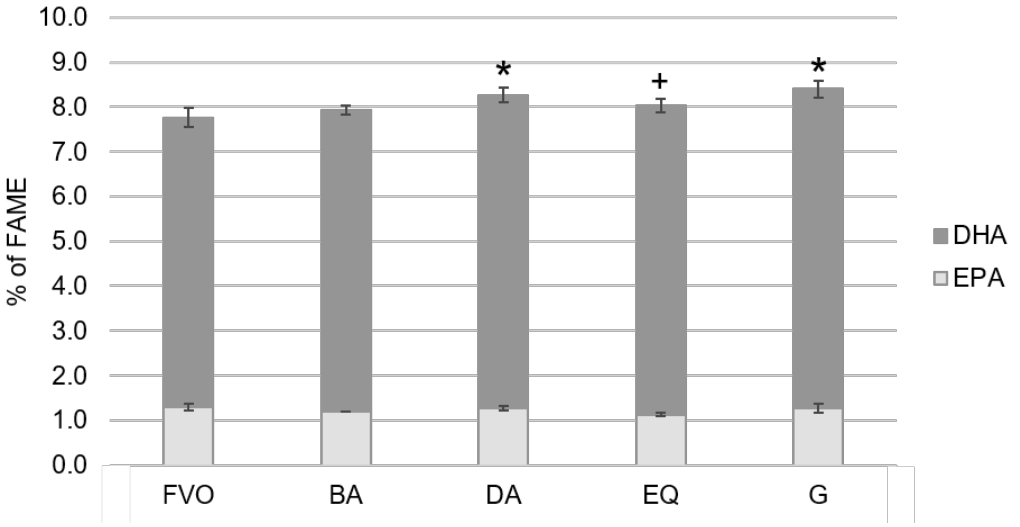

Fig. GD-5 EPA and DHA levels in fillets of rainbow trout fed the experimental diets for eight weeks. All diets were based on a blend of fish and vegetable oils. Control diet (FVO) without supplementation; bioactive substances were added to the diets by 0.15% of DM: BA: biochanin A; DA: daidzein; EQ: equol; G: genistein. Values (mean ± SD, n = 3, consisting of fillets from five fish each) with asterisks are significantly different ($p < 0.05$) from FVO, values without asterisks are not significantly different ($p \geq 0.05$) from FVO. The "+" indicates a tendency towards a statistical difference ($p < 0.1$) in comparison to values of FVO. Values were analyzed according to the statistical methods described in chapter 1 (p. 21).

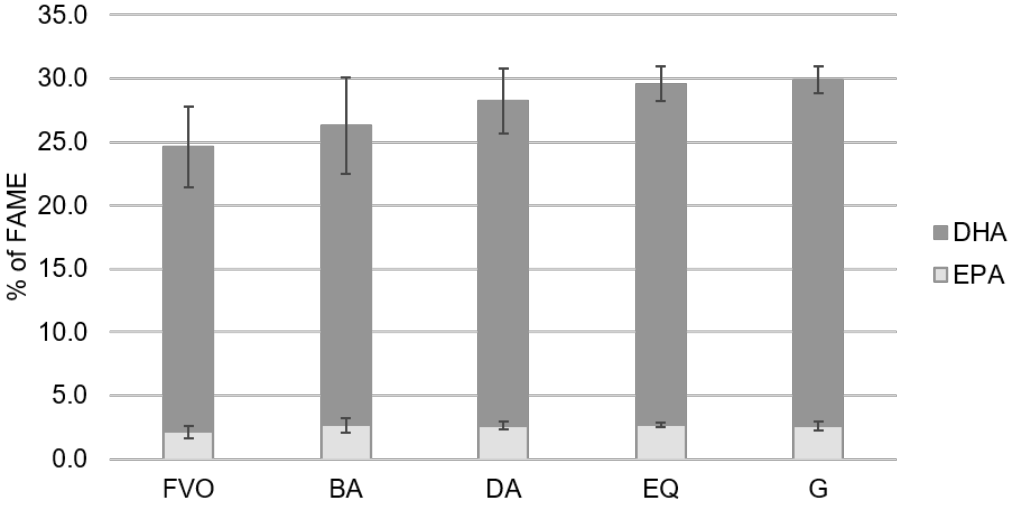

Fig. GD-6 EPA and DHA levels liver samples of rainbow trout fed the experimental diets for eight weeks. All diets were based on a blend of fish and vegetable oils. Control diet (FVO) without supplementation; bioactive substances were added to the diets by 0.15% of DM: BA: biochanin A; DA: daidzein; EQ: equol; G: genistein. Values (mean ± SD, n = 3, consisting of five livers each) without asterisks are not significantly different from FVO ($p \geq 0.05$). Values were analyzed according to the statistical methods described in chapter 1 (p. 21).

Thus, higher dietary SDA levels putatively increase the synthesis rate of EPA and thereby provide more substrate for the synthesis of DHA. Moreover, these findings underline the hypothesis that the first step of the biosynthesis can be bypassed by providing dietary SDA instead of ALA (see Fig. GD-1, p. 103). Thus, a higher share of the key enzyme delta-6-desaturase could be available to convert 24:5n-3 to 24:6n-3 instead of ALA to SDA. Additionally, equol might affect the genes centrally involved in the LC-PUFA biosynthesis via

the aforementioned mechanisms. In turn, equol might have increased the activity of enzymes involved in the biosynthesis in the experiment of **chapter 2** (not measured) but did not increase DHA tissue levels as the substrate for the conversion was limited. Nevertheless, the liver EPA and DHA levels were affected to a greater extent than the fillet levels of fish fed with the Ahiflower oil-based diet with equol in **chapter 4**. This is in accordance with literature data of feeding vegetable oils to salmonids. These studies report that, although the LC-PUFA biosynthesis in the liver of fish is significantly increased, fillet LC-PUFA levels are significantly lower than those of fish fed with a fish-oil based diet [24–26]. However, the results of **chapter 4** indicate that the endogenous biosynthesis is able to compensate for the lack of dietary DHA if a diet based on Ahiflower oil is combined with equol (0.3%). Rainbow trout fed this diet showed similar DHA levels to fish who were fed the fish oil-based diet. However, the levels of EPA were significantly lower, as EPA might have been largely converted to DHA. With regard to the influence of the substrate amount on the biosynthesis efficiency, it might be beneficial to combine equol with a diet containing high amounts of Ahiflower oil and thus ALA and SDA, similar to the diet in **chapter 3**. Thereby, both EPA and DHA could be increased in the fillet and might even exceed the values of fish fed with a fish oil-based control diet.

2. Evaluation of the approaches and conclusion

As mentioned in the introduction, a compound aquafeed has to ensure optimal physical and chemical quality adapted to the fish to achieve a high product quality for human nutrition. Further, it has to be ecologically and economically reasonable. This thesis investigated three approaches to substitute dietary fish oil with vegetable oils and increase the LC-PUFA levels in rainbow trout. As shown in table GD-2, all strategies had no negative effect on the growth of rainbow trout. Further, the crude protein and crude lipid content of whole body homogenates was not affected. In the case of vegetable oils in salmonid diets, these findings are in accordance with recent literature [39]. In contrast, many bioactive substances have been found to impair the growth of fish [56,58,59] and tend to reduce the body lipid content [60,61]. With regard to these findings, the substances and oils used in the experiments of this thesis seem to be suitable for rainbow trout feeds. However, their potential to increase EPA and DHA tissue levels of rainbow trout to achieve a high product quality for human nutrition is less clear.

Among all tested substances, only genistein and equol showed an increase in DHA of 8% in whole body homogenates but not in fillets (**chapter 1**) and did not affect DHA tissue levels when dietary LC-PUFA levels were lower (**chapter 2**). Furthermore, the combination of Ahiflower oil and equol resulted in DHA fillet levels that were increased by 7% in comparison to the Ahiflower oil control group and similar to levels of fish who were fed the fish oil-based diet (**chapter 4**). However, other studies report that bioactive substances such as resveratrol increased DHA levels by 43-71% [59,60] and sesamin by up to 37% in tissues of rainbow trout

[62]. Considering the fact that isoflavones are relatively expensive (equol: 1.90 US$/g), the utilization of these substances in order to increase LC-PUFA levels in the fillets of fish seems economically questionable at this point.

Table GD-2 Overview of main results of chapters 1-4 and additional results.

	Isoflavones Chapter 1 and 2	**Ahiflower oil** Chapter 3	**Combination** Chapter 4
Growth	No effect	Increase final body weight (A100)	No effect
Nutrient composition	No effect	No effect	No effect
EPA and DHA levels			
Liver	No effect	EPA: increase DHA: increase (A66, A100)	EPA: increase (due to Ahiflower oil) DHA: increase (equol 0.2, 0.3%)
Whole body	**Chapter 1**: EPA: no effect DHA: increase (genistein, equol) **Chapter 2**: no effect	Not investigated	EPA: no effect DHA: increase (equol 0.2, 0.3%)
Fillet	**Chapter 1 and 2**: no effect **Additional (FVO)**: DHA: increase (Fig. GD-5)	EPA: increase (A66, A100) DHA: no effect	EPA: Decrease DHA: Similar levels as FV-C
Hepatic mRNA steady state levels			
fads2a(d6)	Decrease (equol)	No effect	No effect
fads2b(d5)	Not investigated	No effect	Not investigated
cpt1a	No effect	No effect	Not investigated
cpt1c	No effect	Decrease (A66, A100)	Not investigated
Fads2a(d6) protein level	No effect	Not investigated	Not investigated

In addition, the impact of bioactive substances on the lipid metabolism might be affected by manifold parameters such as the factors fish size, species and gender [56]. In this thesis, juvenile all-female rainbow trout were used for the experiments. In general, juvenile rainbow trout have a greater capacity to synthesize LC-PUFA endogenously. In addition, female rainbow trout contain 2 to 3 times higher amounts of estrogen receptors than male fish of this species [63]. Thus, the impact on the LC-PUFA biosynthesis particularly of equol, which

putatively developed estrogen-like effects in rainbow trout (**chapters 2** and **4**), might be lower in juvenile and male rainbow trout. Furthermore, the bioavailability of dietary isoflavones is low [64–66] and most of the dietary content might be excreted. Thus, further research would be required to investigate whether the excreted substances accumulate in recirculating aquaculture systems (RAS) and whether they could have an impact on the sexual differentiation and the gonadal development of wild fish populations when discharged via the effluent water.

The aim of this thesis was to find an adequate alternative to dietary fish oil that leads to a high product quality for human nutrition. Thus, the utilization of Ahiflower oil in rainbow trout diets seems promising, as it exceeded the EPA fillet levels of fish which were fed the fish oil-based control diet. Further, the total n-3 levels were significantly increased in the fillets of these fish (**chapter 3**). The increase in ALA and SDA and the simultaneous decrease of linoleic acid and arachidonic acid increased the n-3:n-6 ratio in fillet of rainbow trout fed this diet. This could be also relevant in terms of human nutrition. The consumption of n-3 PUFA rich food might lower the risk of certain chronic diseases and could exert a protective effect on some forms of cancer in humans [72-75]. At the moment, the availability and cost of Ahiflower oil (36 US$/kg) is economically not viable for aquaculture. Therefore, an increased production would be necessary to lower the price. In addition, an extended experimental period might further increase the LC-PUFA fillet levels of fish fed with Ahiflower oil-based diets (**chapter 3**) and the combination of Ahiflower oil and equol (**chapter 4**). The synthesis of LC-PUFA, particularly DHA, can be considered as slow. It might be possible that the rainbow trout from **chapters 3** and **4** needed the entire experimental period to compensate reduced dietary DHA levels and additional time is needed to further increase the DHA levels.

Beside the approach to stimulate the endogenous LC-PUFA biosynthesis in fish, there are other alternatives to substitute dietary fish oil. Although the use of genetically modified oilseed crops and microorganisms has been criticized, it might be a feasible option for the future. Genetically modified plant oils might constitute the most inexpensive source of LC-PUFA as it is estimated that the availability and the costs might be similar to those of oils currently used in aquafeeds [67]. Moreover, due to novel production techniques in the future, alternatives like single cell oils could also be produced in commercially viable amounts [68]. Another potential LC-PUFA source that has been used only minimal so far is krill oil. In 2014, only 0.3 million metric tons were caught in the Antarctic fishing areas [69], although the biomass of krill in the Southern Ocean is estimated to be 700 million metric tons [68]. However, krill is a main component at the base of the food web and thus, overfishing might adversely affect the natural food chain [8,70]. In addition to that, the more efficient use of by-catch and processing by-products to produce fish oil might be an alternative [8]. By that, part of the fish oil used for

aquafeeds can be “recycled”. However, PCBs and dioxins could thereby be accumulated and the risk to transmit diseases would be increased [71].

In conclusion, this thesis showed that the option to increase LC-PUFA fillet levels via increasing the endogenous biosynthesis is possible but only to a limited extent and so far, not economically viable. In addition, future studies should evaluate such strategies over a longer experimental period, ideally over a whole production cycle, as the capacity of the biosynthesis decreases with increasing fish size.

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SUMMARY

Omega-3 (n-3) fatty acids play an important role in human health. Particularly, the fatty acids eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) are known for their health-benefits. For example, EPA has an anti-inflammatory potential and DHA is inter alia crucial for the neuronal development. Humans are able to synthesize EPA and DHA endogenously from the essential precursor fatty acid α-linolenic acid (18:3n-3, ALA). However, this biosynthesis is limited and we rely on the additional dietary uptake of the long-chain polyunsaturated fatty acids (LC-PUFA) EPA and DHA. Fish is the most important source of these fatty acids in human nutrition. The LC-PUFA content in fish depends inter alia on the species and the dietary fatty acid composition. In contrast to most marine fish species, many freshwater fish like rainbow trout (*Oncorhynchus* mykiss) are capable of synthesizing EPA and DHA endogenously. However, similar to humans, the efficiency of this synthesis is limited. In order to ensure a high nutritional quality of aquaculture products for human consumption, significant amounts of these fatty acids need to be provided via the feed. In the past, this has been realized mainly by including marine ingredients like fishmeal and fish oil into the diets. However, due to the continuous growth of the aquaculture sector and the concomitant risen demand for marine feed ingredients, the price of these products increased significantly. Consequently, vegetable oils have increasingly been used as a substitute for fish oil in aquafeeds for salmonids. Common vegetable oils are less expensive than fish oil and readily available but lack highly unsaturated n-3 fatty acids. As already mentioned, the dietary fatty acid composition has a great influence on the fillet fatty acid composition. Thus, by using dietary vegetable oils, the fillet fatty acid composition is adversely modified. Lower EPA and DHA levels in the diet and thus, in the fish, ultimately reduce the product quality of the fillet for human consumption. In order to ensure the supply of EPA and DHA for human nutrition via fish, the identification of alternative feed ingredients, which are equivalent to fish oil, is of the utmost importance. However, potential feed ingredients such as oils of primary producing microalgae and genetically modified plant oils containing significant amounts of EPA and DHA are either not available on a commercial scale yet or their utilization is controversial. The aim of this work was, therefore, to evaluate approaches to enhance the biosynthesis of LC-PUFA and thereby increase EPA and DHA levels in rainbow trout.

The endogenous biosynthesis of LC-PUFA is complex and can be regulated by different factors such as dietary fatty acids and hormones. The use of dietary bioactive substances like isoflavones and their metabolite equol seems to be an appropriate tool to stimulate the endogenous LC-PUFA biosynthesis in rainbow trout. Isoflavones occur naturally in plants like soy (*Glycine max*) and red clover (*Trifolium pratense*) and have characteristics that could affect the biosynthesis via different mechanisms. For example, they have a structural similarity to the hormone estrogen and are further potential ligands for transcription factors of the biosynthesis

(e.g. PPARα). Therefore, they may be able to increase the expression of genes encoding proteins centrally involved in the biosynthesis of LC-PUFA. So far, little is known about the potential and mode of action of these substances on the lipid metabolism in fish in vivo.

Thus, the experiment in **chapter 1** investigated four different isoflavones to identify those which stimulate the LC-PUFA biosynthesis in rainbow trout. For this experiment, juvenile rainbow trout were fed five diets based on a blend of vegetable oils for eight weeks. Four of these diets were supplemented with 0.15% of dry matter of the isoflavones biochanin A, daidzein, genistein and an isoflavone metabolite equol, respectively. A diet without the supplementation served as a control diet. Among all tested substances, only fish which were fed the diets containing equol and genistein showed increased DHA levels in whole body homogenates. In contrast, fillet and liver EPA and DHA levels were not modified by the dietary treatments. Equol might have enhanced the biosynthesis of DHA via estrogen-like mechanisms of action. A fact, which supports this hypothesis, is the increase of the fatty acid 18:0 (stearic acid) in the livers of these fish. An increase of this fatty acid has been associated with increasing estrogen levels in rats. In contrast, genistein could have increased DHA levels in whole body homogenates due to its antioxidative characteristics or via binding to transcription factors. For a better understanding of the mechanisms of action of these substances, potential effects on the hepatic lipid metabolism were investigated. The hepatic mRNA steady state levels and the protein content of the delta-6-desaturase, a key-enzyme of the LC-PUFA biosynthesis, were of particular interest. However, the increase in DHA levels was not supported by the hepatic mRNA steady state levels of the delta-6-desaturase as equol showed decreased levels. In addition, the substances did not affect the protein content of this enzyme. Furthermore, the effects on growth and nutrient composition of fish are important aspects to evaluate the potential of these substances as feed ingredients. These two factors were also not affected by the diets. Thus, these substances can be included at concentrations of 0.15% of dry matter into diets for rainbow trout without impairment of performance parameters. Taken together, equol and genistein seem to enhance the DHA levels in whole body homogenates of rainbow trout, however only to a moderate extent.

Several studies reported that bioactive substances can develop dose-dependent effects in the organism. Thus, different dietary concentrations could increase the stimulation of the LC-PUFA biosynthesis. For that, **chapter 2** investigated whether there is a dose-response of dietary equol and genistein on the LC-PUFA tissue levels in rainbow trout. In this experiment, juvenile rainbow trout were used and fed with a total of seven diets over a period of eight weeks. A vegetable oil-based diet served as a control diet and was supplemented with equol and genistein, respectively at 0.1%, 0.2% and 0.3% of dry matter. An example for a dose-response could be the growth impairment of fish due to increasing dietary concentrations of bioactive substances. This effect has been observed in some studies with rainbow trout and Atlantic

salmon. In the current study, however, no effects of higher concentrations of the respective substance on the growth of fish were found. However, these results were obtained in fish fed on a fixed daily feeding ratio of 1.6% of biomass and might be different if feed intake was higher. Similar to **chapter 1**, rainbow trout fed the equol diets in **chapter 2** showed increased liver levels of stearic acid. Additionally, the liver weights of these fish were increased. This indicates an estrogen-like effect of equol in the liver and thus, underlines the assumptions of **chapter 1**. However, there were no effects of the substances on liver, fillet and whole body homogenate LC-PUFA levels. The diets in **chapter 1** had higher levels of EPA and DHA than those used in **chapter 2**. Thus, it might be possible that diets low in LC-PUFA already fully exploit the endogenous LC-PUFA biosynthesis capacity of rainbow trout and isoflavones are not able to further stimulate the biosynthesis. In conclusion, based on the generated results of **chapter 1** and **2**, the utilization of equol and genistein in diets based on common vegetable oils in order to increase the LC-PUFA fillet levels of rainbow trout seems only possible to a limited extent.

However, there are also other possibilities to stimulate the biosynthesis in rainbow trout. For example, dietary fatty acids can regulate the expression of genes involved in the biosynthesis. Due to a negative feedback mechanism, the absence of LC-PUFA can increase the expression of these genes and thus, stimulate the synthesis of EPA and DHA in fish. In addition, an increased substrate availability of the precursor fatty acids might further increase the overall efficiency of the biosynthesis. Studies in humans, dogs and mice indicate that stearidonic acid (18:4n-3, SDA) is a superior substrate for the synthesis of EPA than ALA, because the first step of the biosynthesis, the delta-6-desaturation of ALA to SDA, can be bypassed.

Thus, **chapter 3** focused on the question whether higher dietary ALA and SDA levels increase the efficiency of the EPA and DHA biosynthesis in rainbow trout. For that, an oil of the plant *Buglossoides arvensis* (Ahiflower oil) was used. Contrasting commercial plant oils, this oil contains high amounts of SDA but is also rich in ALA. In the experiment described in **chapter 3**, juvenile rainbow trout received four diets over a period of eight weeks. A diet based on a blend of fish and vegetable oils served as a control diet. The oils of this diet were substituted with Ahiflower oil at levels of 33%, 66% and 100%. The higher levels of ALA and SDA in the 66% and 100% Ahiflower oil diets resulted in a significant increase of EPA levels in the fillets of fish compared to levels of fish which were fed the control diet. This is particularly relevant because the Ahiflower diets had significantly lower EPA levels than the control diet. This result suggests that a higher substrate availability of precursor fatty acids (ALA and SDA) can increase the efficiency of LC-PUFA synthesis. The biosynthesis of EPA requires a delta-5-desaturation step. For example, Atlantic salmon fed with high dietary SDA levels showed an increased mRNA expression of the delta-5-desaturase. However, the hepatic mRNA steady state levels of the delta-5-desaturase of rainbow trout in this experiment showed no significant

differences among dietary treatments. Furthermore, it seems that high dietary levels of ALA and SDA could compensate for low dietary EPA and DHA levels because the levels of DHA in the fish fillets were not significantly different. In addition, rainbow trout fed the highest dietary Ahiflower oil contents had the significantly highest body weights. These results highlight the potential of Ahiflower oil as a potential candidate to substitute fish oil in rainbow trout diets.

In **chapter 1**, equol increased DHA in whole body homogenates, whereas in **chapter 3** Ahiflower oil increased EPA levels in fillets of rainbow trout. However, the aim of the thesis was to increase both EPA and DHA in fillets of rainbow trout. Thus, the experiment in **chapter 4** aimed to investigate the interactions of dietary equol and Ahiflower oil to improve the fillet quality of rainbow trout. For this experiment, a diet based on a blend of fish and vegetable oils was used as a control diet. In addition, there were four diets based on a blend of Ahiflower oil and vegetable oils. Three of these diets were supplemented with equol in amounts of 0.1%, 0.2% and 0.3% of the dry matter of the diet. Similar to **chapter 2**, no effects on growth and nutrient composition of whole body homogenates of juvenile rainbow trout were observed. In contrast, fish fed with Ahiflower-equol diets showed increased liver weights and higher levels of stearic acid in the liver. This, again, underlines the assumption that equol could develop estrogen-like effects in the liver of rainbow trout. The initial hypothesis that equol could also increase the expression of the target genes of biosynthesis via estrogen-like mechanisms of action, however, could not be proven. Similar to **chapter 1**, there were no significant differences in hepatic mRNA steady state levels of the delta-6-desaturase among treatments. Thus, the increase of DHA levels in the liver of fish fed with the Ahiflower-equol diets cannot be explained by an increased gene expression of delta-6-desaturase. Another possibility could be that equol enhances the activity of this desaturase and a transcription factor centrally involved in the biosynthesis, respectively, by binding to estrogen receptors. However, it seems that equol combined with Ahiflower oil particularly affects the fatty acid composition of the liver. Thus, this combination significantly increased the LC-PUFA levels in this tissue. By comparison, the fillets of fish which were fed diets containing 0.2% and 0.3% equol supplementation, however, had lower EPA levels than fish which were fed the fish oil-based control diet, but both sets had similar DHA levels. Nevertheless, DHA synthesis can be considered as relatively slow and it may require additional time to exceed the DHA levels of the fish fed with the fish oil-based control diet. The fact that equol showed an effect on the LC-PUFA levels in **chapter 4** but not in **chapter 2**, could be due to the dietary SDA content, which could probably have contributed to an increased efficiency of the biosynthesis.

In conclusion, this thesis showed that the endogenous biosynthesis of LC-PUFA in rainbow trout can be regulated by dietary fatty acids and the isoflavone metabolite equol. However, the approach to increase the LC-PUFA fillet levels via the stimulation of the endogenous biosynthesis in order to increase the product quality for human consumption is limited. Further,

it seems that the efficiency of equol and genistein depends on the dietary fatty acid composition. These substances do not have any beneficial effect if dietary EPA and DHA levels are low and simultaneously only ALA but not SDA is provided via the diet. Future studies should investigate the use of Ahiflower oil and equol to increase the LC-PUFA biosynthesis over a longer experimental period, ideally over an entire production cycle, to evaluate the full potential of these ingredients.

ZUSAMMENFASSUNG

Omega-3 (n-3) Fettsäuren sind von großer Bedeutung für die menschliche Gesundheit. Insbesondere die Fettsäuren Eicosapentaensäure (EPA, 20:5n-3) und Docosahexaensäure (DHA, 22:6n-3) sind für ihre potentiell gesundheitsfördernden Effekte bekannt. Während EPA entzündungshemmend wirken kann, ist DHA unter anderem für die neuronale Entwicklung von entscheidender Bedeutung. Der menschliche Organismus ist in der Lage, EPA und DHA aus der essentiellen Vorläufer-Fettsäure α-Linolensäure (ALA, 18:3n-3) endogen zu synthetisieren. Diese Biosynthese findet jedoch nur in begrenztem Maße statt. Deshalb ist der Mensch auf die zusätzliche Aufnahme der langkettigen mehrfach ungesättigten Fettsäuren (LC-PUFA, long-chain polyunsaturated fatty acids) EPA und DHA über die Nahrung angewiesen. Dabei ist Fisch die bedeutsamste Quelle dieser Fettsäuren in der Humanernährung. Der LC-PUFA-Gehalt in Fischen hängt unter anderem stark von der jeweiligen Fischart und der Zusammensetzung der Fettsäuren in der Nahrung ab. Im Gegensatz zu den meisten Seewasserfischen können viele Süßwasserfische wie die Regenbogenforelle (*Oncorhynchus mykiss*), EPA und DHA endogen synthetisieren. Ähnlich wie beim Menschen ist die Effizienz dieser Synthese jedoch begrenzt. Um eine hohe Produktqualität von Aquakulturerzeugnissen für den menschlichen Verzehr zu gewährleisten, müssen folglich erhebliche Mengen dieser Fettsäuren über das Fischfutter bereitgestellt werden. In der Vergangenheit wurde dies vor allem über den Einsatz von marinen Rohstoffen wie Fischmehl und Fischöl in der Fischernährung umgesetzt. Durch das kontinuierliche Wachstum des Aquakultursektors und dem damit einhergehenden zunehmenden Bedarf an marinen Rohstoffen, stieg der Preis für diese Produkte deutlich an. Deshalb wurden vermehrt pflanzliche Öle als Substitut für Fischöl in Futtermitteln für Salmoniden eingesetzt. Pflanzenöle sind günstiger als Fischöl und besser verfügbar, beinhalten jedoch außer ALA keine längerkettigen n-3 Fettsäuren. Wie bereits erwähnt hat die Fettsäurezusammensetzung des Futtermittels einen beträchtlichen Einfluss auf die Fettsäurezusammensetzung des Fischfilets. Durch die Verwendung von Pflanzenölen im Futtermittel wird somit die Fettsäurezusammensetzung des Filets nachteilig modifiziert. Durch die geringeren EPA- und DHA-Gehalte sowohl im Futter als auch dann im Fisch wird letztlich die Produktqualität des Filets für den menschlichen Verzehr reduziert. Um die Bereitstellung an EPA und DHA über das Lebensmittel Fisch für die Humanernährung gewährleisten zu können, ist die Identifikation alternative Rohstoffe, die ein Äquivalent zu Fischöl darstellen, von immenser Bedeutung. Potentielle Futtermittelrohstoffe wie Öle von primärproduzierenden Mikroalgen und gentechnisch veränderte Pflanzenöle, die EPA und DHA in nennenswerten Mengen enthalten, sind allerdings entweder noch nicht kommerziell erhältlich oder in ihrer Verwendung umstritten. Ziel der vorliegenden Arbeit war es deshalb, Möglichkeiten zur Steigerung der Synthese von

LC-PUFA zu evaluieren, um die Gehalte an EPA und DHA in der Regenbogenforelle zu erhöhen.

Die endogene Biosynthese von LC-PUFA ist komplex und kann durch verschiedene Faktoren wie beispielsweise Nahrungsfettsäuren und Hormone reguliert werden. Die Verwendung von bioaktiven Substanzen, wie Isoflavone und der Isoflavon-Metabolit Equol, scheint ein geeignetes Mittel zur Stimulierung der endogenen LC-PUFA-Biosynthese in der Regenbogenforelle zu sein. Isoflavone kommen natürlicherweise in Pflanzen wie Soja (*Glycine max*) und Rotklee (*Trifolium pratense*) vor und besitzen Eigenschaften, die die Biosynthese über verschiedene Mechanismen beeinflussen können. Zum Beispiel haben sie eine strukturelle Ähnlichkeit mit dem Hormon Östrogen und sind außerdem potentielle Liganden für Transkriptionsfaktoren der Biosynthese (z.B. PPARα). Dadurch könnten sie möglicherweise die Expression von den Genen erhöhen, die zentral an der LC-PUFA Biosynthese beteiligt sind. Bislang ist allerdings wenig über das Potential und die Wirkweise dieser Substanzen auf den Lipidmetabolismus in Fischen bekannt.

Daher wurden in **Kapitel 1** vier verschiedene Isoflavone hinsichtlich ihres Potentials, die LC-PUFA-Biosynthese in Regenbogenforellen zu stimulieren, untersucht. Für dieses Experiment wurden fünf Diäten basierend auf einer Mischung aus Pflanzenölen konzipiert und über einen Zeitraum von acht Wochen an juvenile Regenbogenforellen verfüttert. Vier dieser Diäten wurden mit 0,15% der Isoflavone Biochanin A, Daidzein, Genistein bzw. Equol supplementiert. Die fünfte Diät, ohne Zusatz, diente als Kontrolldiät. Von allen getesteten Substanzen zeigten nur Fische, die mit Equol und Genistein gefüttert wurden, erhöhte DHA-Werte im Ganzkörperhomogenat. Im Gegensatz dazu wurden die EPA- und DHA-Gehalte im Filet und in der Leber nicht durch die Futtermittel beeinflusst. Equol könnte die Biosynthese von DHA über östrogen-ähnliche Wirkmechanismen gesteigert haben. Ein Fakt, der diese These stützt, ist die Erhöhung der Fettsäure 18:0 (Stearinsäure) in der Leber dieser Fische. Ein Anstieg dieser Fettsäure wurde mit steigenden Östrogenspiegeln bei Ratten in Verbindung gebracht. Im Gegensatz dazu könnte Genistein den DHA-Gehalt im Ganzkörperhomogenat aufgrund der antioxidativen Eigenschaften oder mittels Bindung an Transkriptionsfaktoren erhöht haben. Zum besseren Verständnis der potentiellen Wirkmechanismen dieser Stoffe, wurden mögliche Auswirkungen auf den Lipidstoffwechsel der Leber untersucht. Von besonderem Interesse waren hierbei die hepatische mRNA-Level und der Proteingehalt der Delta-6-Desaturase, einem Schlüsselenzym der LC-PUFA-Biosynthese. Der Anstieg des DHA-Gehalts wurde jedoch nicht durch die Ergebnisse der Genexpressionsanalyse gestützt, da die Fische, die mit der Equol-Diät gefüttert wurden sogar verringerte mRNA-Level aufwiesen. Außerdem konnte kein Einfluss der Substanzen auf die Proteinmenge dieses Enzyms nachgewiesen werden. Ein weiterer Aspekt, der wichtig für den potentiellen Einsatz als Futtermittelrohstoff ist, ist die Wirkung der Substanzen auf das Wachstum und die

Nährstoffzusammensetzung der Fische. Diese beiden Faktoren wurden ebenfalls nicht durch die Diäten beeinflusst. Somit können diese Substanzen in Konzentrationen von 0,15% der Trockensubstanz in der Diät für Regenbogenforellen verwendet werden, ohne die Leistungsparameter zu beeinträchtigen. Equol und Genistein können die DHA-Gehalte im Ganzkörperhomogenat von Regenbogenforellen erhöhen, jedoch nur in einem geringen Ausmaß.

Studien mit bioaktiven Substanzen deuten darauf hin, dass diese Stoffe im Organismus dosisabhängige Wirkungen entfalten können. Daher könnten unterschiedliche diätetische Konzentrationen zu einer verstärkten Stimulation der LC-PUFA-Biosynthese in Regenbogenforellen führen. Deshalb wurde in **Kapitel 2** untersucht, ob es eine Dosis-Wirkungs-Beziehung von Equol und Genistein auf die LC-PUFA-Gehalte im Gewebe von Regenbogenforellen gibt. In diesem Experiment wurden juvenilen Regenbogenforellen verwendet und mit insgesamt sieben Diäten über eine Dauer von acht Wochen gefüttert. Eine auf Pflanzenöl basierende Diät diente als Kontrolldiät und wurde mit Equol bzw. Genistein in einer Menge von 0,1, 0,2 und 0,3% der Trockensubstanz der Diät ergänzt. Ein Beispiel für einen Dosis-Wirkungs-Zusammenhang wäre die Beeinträchtigung des Wachstums mit zunehmender Konzentration der bioaktiven Substanz im Futtermittel. Dieser Effekt wurde in einigen Studien mit Regenbogenforellen und dem Atlantischen Lachs beobachtet. In der aktuellen Studie wurden jedoch keine Auswirkungen einer höheren Konzentration des jeweiligen Stoffs auf das Wachstum der Fische gefunden. Dieser Befund ist allerdings auf das tägliche Fütterungsniveau von 1.6% der Biomasse der Fische beschränkt und schließt nicht aus, dass es bei einer höheren Futteraufnahme zu einer Beeinträchtigung des Fischwachstums kommen kann. Ähnlich zu den Ergebnissen aus **Kapitel 1** wiesen Regenbogenforellen, die mit Equol gefüttert wurden, in der Studie in **Kapitel 2** höhere Stearinsäuregehalte in der Leber auf. Zusätzlich waren bei diesen Fischen die Lebergewichte erhöht. Dies deutet auf eine östrogen-ähnliche Wirkung von Equol in der Leber hin und unterstreicht somit die Annahme aus **Kapitel 1**. Im Gegensatz dazu gab es keine Auswirkungen der Substanzen auf die LC-PUFA-Gehalte in der Leber, im Filet und im Ganzkörperhomogenat der Fische. Die Diäten in **Kapitel 1** hatten höhere EPA- und DHA-Gehalte im Vergleich zu den in **Kapitel 2** verwendeten Diäten. Daher könnte es möglich sein, dass geringe LC-PUFA Gehalte im Futtermittel die endogene LC-PUFA-Biosynthesekapazität der Fische bereits voll ausschöpfen und eine weitere Stimulation mittels Isoflavonen nicht möglich ist. Auf Grund der nicht vorhandenen bis geringen Wirkung erscheint die Verwendung von Equol und Genistein in Diäten auf der Basis kommerziell eingesetzter Pflanzenöle zur Erhöhung der LC-PUFA-Gehalte im Filet von Regenbogenforellen scheint nur in begrenztem Umfang möglich.

Es gibt jedoch auch andere Möglichkeiten, die Biosynthese in Regenbogenforellen zu stimulieren. Zum Beispiel können Fettsäuren, die über die Nahrung aufgenommen wurden, die Expression von Genen, die maßgeblich an der Biosynthese beteiligt sind, regulieren. Über einen negativen Feedback-Mechanismus kann die Abwesenheit von LC-PUFA die Genexpression eben dieser Gene erhöhen und somit die Synthese von EPA und DHA im Fisch stimulieren. Darüber hinaus könnte eine Steigerung der Substratmenge der Vorläufer-Fettsäuren die Gesamteffizienz der Biosynthese weiter erhöhen. Studien mit Menschen, Hunden und Mäusen zeigen, dass Stearidonsäure (SDA, 18:4n-3) für die Synthese von EPA ein höherwertigeres Substrat als ALA sein kann, da der erste Schritt der Biosynthese – die Delta-6-Desaturierung von ALA zu SDA – übersprungen werden kann.

Daher sollte in **Kapitel 3** untersucht werden, ob höhere ALA- und SDA-Gehalte im Futtermittel die Effizienz der endogenen EPA- und DHA-Biosynthese bei Regenbogenforellen steigern können. Dafür wurde ein Öl der Pflanze *Buglossoides arvensis* (Ahiflower Öl) verwendet. Im Gegensatz zu herkömmlichen Pflanzenölen enthält dieses Öl hohe Mengen an SDA, ist aber ebenfalls reich an ALA. Im Experiment, das in **Kapitel 3** beschrieben wird, erhielten juvenile Regenbogenforellen über einen Zeitraum von acht Wochen vier Futtermittel. Als Kontrolle diente eine Diät, die auf einer Mischung aus Fischöl und pflanzlichen Ölen basierte. Dieses Ölgemisch wurde zu 33, 66 und 100% durch Ahiflower Öl ersetzt. Die höheren Gehalte an ALA und SDA in den Futtermitteln mit 66 und 100% Ahiflower Öl-Anteil führten zu einem signifikanten Anstieg des EPA-Gehalts im Forellenfilet im Vergleich zu den Fischen, die mit der Kontrolldiät gefüttert wurden. Dies ist besonders relevant, da die Ahiflower-Diäten deutlich geringere EPA-Gehalte als die Kontrolldiät aufwiesen. Dieses Ergebnis deutet darauf hin, dass eine größere Substratmenge an Vorläufer-Fettsäuren (ALA und SDA) tatsächlich die Effizienz der LC-PUFA Synthese steigern kann. Für die Biosynthese von EPA wird ein Delta-5-Desaturaseschritt benötigt. So wiesen Lachse, die mit hohen SDA-Gehalten im Futtermittel gefüttert wurden, eine gesteigerte mRNA-Expression der Delta-5-Desaturase auf. Die mRNA-Level der Delta-5-Desaturase der Fische dieses Versuchs zeigten jedoch keine signifikanten Unterschiede zwischen den verschiedenen Futtergruppen. Da sich außerdem die DHA-Gehalte der Fischfilets nicht signifikant voneinander unterschieden, scheint es, als könnten hohe Gehalte an ALA und SDA die geringen EPA und DHA Gehalte in der Diät kompensieren. Darüber hinaus wiesen die Forellen, die mit den höchsten Konzentrationen an Ahiflower Öl gefüttert wurden, die signifikant höchsten Körperendgewichte auf. Dieses Ergebnis unterstreicht das Potential des Ahiflower Öls als Alternative zum Einsatz von Fischöl in Futtermitteln für Regenbogenforellen.

In **Kapitel 1** erhöhte Equol den DHA-Gehalt im Ganzkörperhomogenat, wohingegen Ahiflower Öl in **Kapitel 3** die EPA-Gehalte im Filet von Regenbogenforellen erhöhte. Ziel dieser Arbeit war es jedoch, sowohl EPA als auch DHA im Filet der Regenbogenforelle zu steigern. Daher

lag der Fokus des Experiments in **Kapitel 4** darauf, eine mögliche Wechselwirkungen von Equol mit Ahiflower Öl zu untersuchen, um die Filetqualität von Regenbogenforellen zu verbessern. Für diesen Versuch wurde ein Futtermittel basierend auf einem Gemisch aus Fischöl und pflanzlichen Ölen als Kontrolldiät verwendet. Zusätzlich gab es vier Diäten, basierend auf einer Mischung aus Ahiflower Öl und pflanzlichen Ölen. Drei dieser Diäten wurden mit Equol in Mengen von 0,1, 0,2 und 0,3% der Trockensubstanz der Diät ergänzt. Ähnlich zu **Kapitel 2** wurden keine Auswirkungen der Equol-Zulage auf das Wachstum von Fischen und die Nährstoffzusammensetzung der Ganzkörperhomogenate der juvenilen Regenbogenforellen festgestellt. Im Gegensatz dazu zeigten Fische, die mit Ahiflower-Equol-Diäten gefüttert wurden, ein erhöhtes Lebergewicht und höhere Gehalte an Stearinsäure in der Leber auf. Dies unterstreicht erneut die Vermutung, dass Equol in der Leber von Regenbogenforellen eine östrogen-ähnliche Wirkung erzeugen könnte. Die Anfangshypothese, dass Equol ebenfalls über östrogen-ähnliche Wirkmechanismen die Expression der Zielgene der Biosynthese steigern könnte, konnte dagegen nicht belegt werden. Auch in diesem Versuch gab es keine signifikanten Unterschiede zwischen den mRNA-Leveln der Delta-6-Desaturase in den Lebern von Fischen der verschiedenen Futtergruppen. Demnach kann der DHA-Anstieg in der Leber der mit Ahiflower-Equol gefütterten Fische nicht über eine erhöhte Genexpression der Delta-6-Desaturase erklärt werden. Eine weitere Möglichkeit wäre, dass Equol die Aktivität dieser Desaturase oder eines Transkriptionsfaktors, der zentral an der Biosynthese beteiligt ist, erhöht, indem es an Östrogenrezeptoren bindet. Es scheint aber, als erziele Equol in Zusammenhang mit Ahiflower Öl vor allem in der Leber maßgebliche Effekte auf das Fettsäuremuster. So steigerte diese Kombination die LC-PUFA-Gehalte signifikant in diesem Gewebe. Im Vergleich dazu wies das Filet der Fische, denen Diäten mit 0,2 und 0,3% Equol Supplementierung gefüttert wurden, allerdings geringere EPA-Gehalte und ähnliche DHA-Gehalte auf, wie diejenigen Fische, die die Fischöl-Kontrolldiät erhielten. Indessen ist die DHA-Synthese relativ langsam und es könnte sein, dass zusätzliche Zeit erforderlich ist, um die DHA-Gehalte der Fische, die mit der Kontrolldiät auf Fischölbasis gefüttert wurden, zu überschreiten. Dass Equol in dieser Studie im Gegensatz zur Studie in **Kapitel 2** einen Effekt auf die LC-PUFA-Synthese zeigt, könnte am diätetischen SDA-Gehalt liegen, der vermutlich zu einer gesteigerten Effizienz der Biosynthese beigetragen haben könnte.

Zusammenfassend zeigt diese Arbeit, dass die endogene Biosynthese von LC-PUFA in Regenbogenforellen durch Fettsäuren in der Nahrung und den Isoflavon-Metaboliten Equol reguliert werden kann. Der Ansatz, die LC-PUFA-Gehalte im Filet durch Stimulierung der endogenen Biosynthese zu steigern, um die Produktqualität für den menschlichen Verzehr zu erhöhen, ist jedoch nur in begrenztem Umfang möglich. Ferner scheint es, dass die Wirksamkeit von Equol und Genistein von der Fettsäurezusammensetzung des Futtermittels

abhängt. Diese Substanzen haben keine Wirkung auf die LC-PUFA-Gehalte in der Forelle, wenn im Futtermittel die EPA- und DHA-Konzentrationen gering sind und gleichzeitig nur ALA, aber kein SDA, im Futtermittel vorhanden ist. Zukünftige Studien sollten den Einsatz von Ahiflower Öl und Equol zur Steigerung der LC-PUFA-Biosynthese über einen längeren Versuchszeitraum, idealerweise über einen gesamten Produktionszyklus, untersuchen, um das gesamte Potential dieser Rohstoffe zu bewerten.

APPENDIX

Nutrient composition

Used in chapter 1-4

The proximate nutrient analysis was conducted for samples of experimental diets, fillets and whole body homogenates. It was performed at the laboratory of the Gesellschaft für Marine Aquakultur according to EU guideline (EC) 152/2009 [1]. The analysis included the determination of dry matter (DM), crude protein, crude lipid and crude ash.

Prior to the analysis, fillet and whole body homogenate samples were freeze dried (alpha 1-4 LSC; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) until the weight was constant. Subsequently, these samples were homogenized with a knife mill (Grindomix, Retsch, Haan, Germany). All samples were analyzed in duplicate. For the determination of dry matter and crude ash, samples (2g) were dried at 103°C in a drying oven (ED53 9010-0078; Binder GmbH, Tuttlingen, Germany) until constant mass. After the samples were weighed to determine dry matter, same samples were incinerated for 4 h at 560°C in a muffle furnace (LE 6/11/P300; Nabertherm, Lilienthal, Germany) to determine crude ash. Crude protein content was analyzed according to the methods of Kjeldahl. The samples (0.5g) were prepared via digestion (KjelDigester K-449 and Scrubber K-415; BÜCHI Labortechnik GmbH, Essen, Germany) with sulphuric acid and distillation (KjelFlex 360; BÜCHI Labortechnik GmbH) with sodium hydroxide and boric acid following titration (877 Titrino plus; Deutsche METROHM GmbH & Co. KG, Filderstadt, Germany) using a nitrogen to protein coefficient of 6.25. Crude lipid content was determined according to the Soxhlet method. Samples (2g) were hydrolyzed with hydrochloric acid (HYDROTHERM HT 6; C. Gerhardt GmbH & Co. KG, Königswinter, Germany) and dried prior to the extraction with petroleum ether (SOXTHERM 416, Multistat/SX PC; C. Gerhardt GmbH & Co. KG, Königswinter, Germany). The gross energy content (MJ/kg) was determined by bomb calorimetry (C 200; IKA-Werke GmbH & Co. KG, Staufen, Germany) using 0.5g per sample for each analysis. Prior to the measurement, the device was calibrated with benzoic acid. The content of total carbohydrates was calculated as follows: *Total carbohydrates = 100 – (crude protein + crude lipid + crude ash).*

Fatty acid composition

Used in chapter 1-4

Fatty acid composition of oils, diets, liver, whole body homogenate and fillet samples was analyzed by LUFA-ITL GmbH in Kiel, Germany. The analyses were conducted using gas chromatography (GC) (Deutsche Gesellschaft für Fettwissenschaft e.V. (DGF), C-VI 10a).

Samples (1g) were prepared using saponification with methanolic NaOH and transmethylation of total lipids with boron trifluoride and methanol (DGF, C-VI 11a). The obtained fatty acid methyl ester (FAME) samples were separated by GC via split-injection (column: CP-Sil 88 50 m x 0.25 mm x 0.2 µm or similar) with an injection amount of 0.5 µl and a split ratio of 12.5:1. In addition, FAME were detected using a flame ionization detector (FID) with helium as a carrier gas. The individual FAME were identified by comparison with certified standard mixtures (18919-1AMP Supelco, F.A.M.E. Mix, Sigma-Aldrich) similar to the chromatogram shown in Fig A-1. Finally, the fatty acid composition was calculated as percent of FAME relative to total FAME. If required by the journal (*Lipids*, manuscripts of chapter 3 and 4), FAME values were converted to moles and the fatty acid composition data is reported as mole% of total fatty acids.

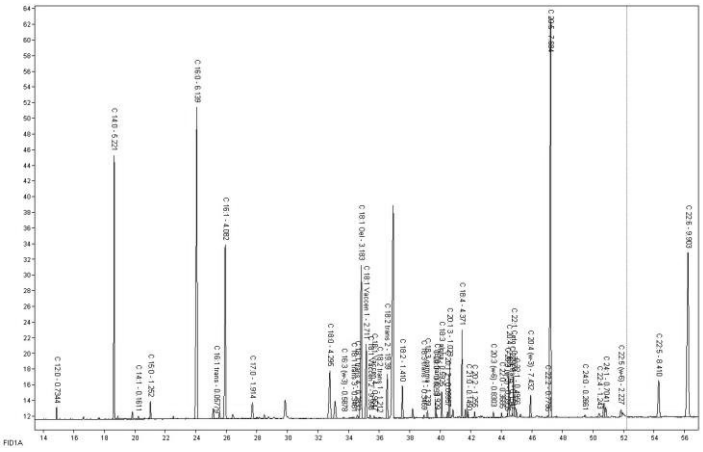

Fig. A-1 Represantive GC chromatogram of fatty acid methyl ester (FAME) from a standard mixture for fish samples. Each peak represents a specific FAME and the peak area is correlated to the total amount of FAME.

RNA isolation and qRT-PCR

Used in chapter 1, 3 and 4

Prior to total RNA isolation, approximately 15 mg of RNA later stabilized liver tissue of rainbow trout was homogenized in a TissueLyser II (Qiagen, Hilden, Germany). Total RNA was extracted from liver samples using the Innuprep RNA Mini Kit (Analytik Jena, Jena, Germany) according to the manufacturer's protocol. RNA concentration and purity were determined by measuring the absorbance at ratios of A260/280 and 260/230 via NanoDrop measurements (NanoDrop2000c; ThermoScientific, Waltham, MA, USA). For the following analysis, each sample was diluted with DEPC treated water to result in a RNA concentration of 100 ng/µl.

Hepatic mRNA steady state levels of selected target genes were quantified via quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) measurements using the SensiFast SYBR No-ROX One-Step Kit (Bioline, London, UK) and a Rotor-Gene 6000 real-time PCR cycler (Corbett/Qiagen). Eighteen microliters of a 1000-fold-diluted RNA sample were added to 27 µl Master Mix. The 27 µl Master Mix consisted of 2.48 µl DEPC treated water,

0.40 µl reverse transcriptase, 0.90 µl RNase inhibitor, 22.5 µl 2x SensiFAST-Mix and 0.36 µl of each primer. The analysis was conducted in duplicate using 20 µl of this mixture each. The cycling conditions were as follows: at 49 °C for 30 min, at 95 °C for 10 min, 40 cycles of 95 °C for 15 s, annealing temperature of the primer pair for 30 s and 72 °C for 30 s, followed by a melt curve analysis from 60 to 99 °C of 1 °C increments. Primer sequences and the respective annealing temperatures are listed in the respective chapters separately. A standard curve was used to calculate relative mRNA concentrations. The mRNA steady state levels of genes encoding proteins related to lipid metabolism were normalized to the expression level of the housekeeping gene elongation factor 1 alpha (*ef1α*). *Ef1α* was previously used and suggested as a gene reference in salmonids [2].

Enzyme-Linked Immunosorbent Assay (ELISA)

Used in chapter 1

The protein levels of Fads2a(d6) were determined using a Fish Fatty Acid Desaturase 2 ELISA Kit (MBS066226, MyBiosource Inc., San Diego, CA, USA; purchased from Biozol, Eching, Germany) according to the manufacturer's protocol. Rainbow trout liver samples diluted in phosphate buffered saline in a TissueLyser II (Qiagen, Hilden, Germany). After the centrifugation, standards and diluted samples were applied to the Microelisa 96-well plate. Samples were incubated and treated with horseradish peroxidase (HRP) conjugate reagent. Wells were sealed following a 60-minute incubation at 37°C in a microplacte rader. Subsequently, wells were washed multiple times. Color intensity was determined at 450 nm using a Labsystems iEMS MF multiplate reader (MTX Lab Systems, Bradenton, FL, USA purchased from Thermo Fisher Scientific, Darmstadt, Germany). The Fads2a(d6) protein concentration in the liver samples of rainbow trout was calculated via standard curve. Values were normalized to the total protein concentration determined by bicinchoninic acid (BCA) measurements. For these measurements, a Pierce BCA Protein Assay Kit was used according to the manufacturer's protocol.

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2. Olsvik PA, Lie KK, Jordal AEO, Nilsen TO, Hordvik I. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. BMC Mol Biol. 2005;6: 1–9. doi:10.1186/1471-2199-6-21

ACKNOWLEDGEMENTS

An dieser Stelle möchte ich mich bei den Personen bedanken, die mich in meiner Arbeit, aber auch allgemein während der Zeit, die ich in Büsum verbracht habe, unterstützt haben.

Carsten, ich möchte dir von ganzem Herzen für deine Betreuung danken. Du hast mich gefördert und auch gefordert und mich angespornt, meine Leistungen weiter zu verbessern. Danke auch für dein Vertrauen, dass ich selbständig arbeiten konnte. Gleichzeitig wusste ich aber, dass ich jederzeit auf dein Feedback und deine Unterstützung zählen kann.

Gerald, vielen lieben Dank für deinen umfangreichen fachlichen Rat und deinen beeindruckenden Ideenreichtum, der meine Arbeit vielfältiger gemacht hat.

Adrian, Bahne, Carsten und Julian – meine lieben Studenten! Ohne euch wäre es vermutlich nicht möglich gewesen, den Versuchs- und Probenumfang dieser Arbeit zu bewältigen. Danke für euren unermüdlichen Einsatz. Ich bin stolz, dass wir das zusammen gemeistert haben.

Steffi, ich weiß es wirklich sehr zu schätzen, dass du dir für mich Zeit genommen hast, obwohl du es nicht hättest machen müssen. Außerdem herzlichen Dank an dich und Sandra, dass ihr die riesigen Probenmengen so schnell bearbeitet habt.

Stephie, danke für deine Unterstützung, meine Arbeit besser zu machen und den Zuspruch, an meine Fähigkeiten zu glauben. Du bist für mich ein Vorbild, nicht nur als exzellente Wissenschaftlerin, sondern auch als wunderbarer Mensch, der in jeder Situation die richtigen Worte findet.

Mario, ich bin dir sehr dankbar für deine Unermüdlichkeit, mir Statistik näherzubringen. Und dass du dich über kleine Erfolge gefreut hast, hat mich unglaublich aufgemuntert.

Natürlich möchte ich mich auch noch bei allen GMAlern bedanken. Ich konnte mich immer auf eure Hilfe bei Versuchsauflösungen und Vorträgen verlassen. Vielen Dank aber auch für schöne Grillabende und gemeinsame Unternehmungen. Mein besonderer Dank gilt dir, Claudia, für deinen fachlichen Rat, der mir wirklich viel geholfen und mir sicherlich viel Mühe erspart hat.

Markus, für deine stets aufmunternden Worte bin ich dir unglaublich dankbar. Du warst immer für mich da, wenn ich was auf dem Herzen hatte und hast mich immer zum Lachen gebracht, wenn mir auch mal nicht danach war.

Jonas danke für deine Gastfreundschaft, deine Hilfe und für ein bisschen Hohenheimer-Studenten-Leben in Kiel.

Katha, Lisa, Tati – auch wenn wir uns nicht mehr oft sehen, ich weiß, dass ihr immer für mich da seid. Danke, dass ich auf auch zählen kann! #BBF #memories # goodtimes

Meine lieben Omas, auch wenn ihr nicht mehr hier seid, habt ihr mir viel geholfen. Oma G., mit deiner Ruhe und Zuversicht warst du mir ein großes Vorbild. Oma F., dein „Da musst du halt auf die Zähne beißen“ hat mich durch viele anstrengende Phasen begleitet.

Mama und Papa, ohne euch wäre ich nicht so weit gekommen. Ihr habt mich bei allem unterstützt, was ich machen wollte. Für eure Liebe und euer Vertrauen möchte ich euch von ganzem Herzen danken. Auch an meine Geschwister Julia, Theresia und Jossi: ich bin immer wieder gerne „daheim“ und genieße jeden Moment mit euch. Danke für alles!

Benni, danke für deine Hilfe, deinen Zuspruch und den Halt, den du mir immer gegeben hast. “I never want to stop making memories with you.“ (Beverly Preston - Holding onto Hope)

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EIGENE NOTIZEN